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HENRY CHANDLER COWLES

(WITH PORTRAIT)

HENRY CHANDLER COWLES was born at Kensington, Connecticut, on February 27, 1869. He was graduated from Oberlin College with the degree of A.B. in 1893, and the next year taught natural science at Gates College. In 1895 he was given a fellowship at the University of Chicago, beginning his graduate studies in geology; but later he became a member of the first group of students in the newly organized Department of Botany of this Institution, and an assistant in that Department in 1897. He received the degree of Doctor of Philosophy in 1898, presenting as his thesis his classical paper *The Ecological Relations of the Vegetation on the Sand Dunes of Lake Michigan*. Application of his concepts concerning dynamic vegetation to vegetation in general resulted in his *Physiographic Ecology of Chicago and Vicinity*, a philosophy relating to vegetation in which the central principle was that classification to be valid must be genetic and dynamic. For the first time the concepts of succession and climax were adequately expressed. The principles expressed have stood the test of time and have formed the basis and point of departure for the dynamic concept of ecology.

In the Department of Botany at the University of Chicago DR. COWLES was repeatedly advanced in rank, becoming Professor in 1911 and Chairman of the Department in 1925. In 1926 he became Editor of the BOTANICAL GAZETTE. To both tasks he gave a full measure of devotion and energy until his retirement in 1934.

In 1911 appeared the *Chicago Textbook of Botany*. In the volume dealing with ecology DR. COWLES stressed the theory of mechanical causation rather than of teleology and adaptation. In 1914 the Eco-

logical Society of America was organized, largely through his efforts and those of his former students. He was its first secretary-treasurer, its president in 1917, and always a wise counselor regarding its welfare. In 1935 the July issue of *Ecology*, filled with articles from students and colleagues throughout the world, was dedicated to him by his students and friends. His alma mater, Oberlin College, conferred upon him the honorary degree of Sc.D. in 1923.

For many years DR. COWLES was president of the Chicago Academy of Sciences, a charter member and an active supporter of the Illinois State Academy of Science, and also a patron and trustee of the Geographic Society of Chicago and president of the Society for a term of years. A member of many other scientific societies, he served as president of the Association of American Geographers in 1910; as president of the Botanical Society in 1922; and vice-president of Section G of the American Association for the Advancement of Science in 1913. In 1930 he was made president of the section of phytogeography and ecology of the International Botanical Congress which met at Cambridge, England.

As a leader in plant science, particularly in dynamic plant ecology, DR. COWLES was enthusiastic but never dogmatic. From the beginning of his career to his retirement there was always gathered about him a group of men and women who aided in augmenting and refining his concepts and in spreading the knowledge of dynamic ecology.

No teacher brought his students more directly to nature than he. Much of his teaching was done on field trips, and he encouraged others to use the same classroom. As a scientist and as a teacher he inspired his colleagues and students in the quest of accomplishment. But in the hearts and minds of his associates there was the love for him personally, as great as the respect for his scientific attainment. Unfailing good humor he had in abundance. To live and to work intimately with him was to come to know and appreciate his sense of rightness, of justice, and his depth of understanding.

HENRY CHANDLER COWLES passed away at his home, September 12, 1939. His achievements laid the foundation for a new and useful branch of science. Through professional and personal contacts he had a multitude of devoted friends. The impress of his having lived is deep in the field of the science he served and in the minds of those who knew him.—E. J. KRAUS.

NUTRITION OF THE MYXOMYCETES. I. PURE CULTURE AND TWO-MEMBERED CULTURE OF MYXOMYCETE PLASMODIA[1]

ARTHUR L. COHEN

(WITH FOUR FIGURES)

Introduction

The Myxogastrales (myxomycetes) and their probable congeners, the Acrasiales, have long attracted attention because of their position at the boundary of the plant and animal kingdoms. They are particularly interesting in their faculty of holozoic nutrition, coupled with the formation of definitely organized fruiting bodies—the latter a plantlike character. The myxomycetes possess the additional experimentally valuable property of forming large ameboid plasmodia, of indefinite extent and of relatively unspecialized nature.

In view of their interest it is surprising that nearly all, if not all, studies on the myxomycetes have been made with contaminated plasmodia, and many of these observations were made somewhat cursorily on plasmodia brought in directly from the field, not too well freed of their natural substrates, and under uncontrolled conditions.

Controlled, reproducible laboratory cultures are of great value for the experimental study of the myxomycetes no less than for other organisms. For extensive nutritional studies, controlled cultures are a *sine qua non*. The work reported in this paper has been on the preparation and maintenance of controlled cultures for the studies on nutrition of the myxomycetes now being carried on in this laboratory.

Literature review

GENERAL OBSERVATIONS.—In spite of the criticism of past work just mentioned, students of the myxomycetes have been able to make cogent observations on the feeding habits of myxomycete

[1] Contribution from the Laboratories of Cryptogamic Botany and the Farlow Herbarium, Harvard University, no. 174.

plasmodia and were often able to draw valuable inferences. The first of these observations were made by CIENKOWSKI and DEBARY.

The ingestion of solid particles was recorded by CIENKOWSKI (**11**) and DEBARY (**1, 2, 3**). Neither could decide whether the chief means of feeding was by the ingestion and assimilation of solid food or by the absorption of dissolved nutrients through the plasma membrane.

That some myxomycete plasmodia can live wholly on ingested particulate matter was shown by LISTER (**21**). He refers to the "parasitism" of *Badhamia utricularis* on hymenophores, particularly *Stereum hirsutum*. He observed the dissolution of the hyphae ingested by the plasmodium as well as a similar destruction of starch grains previously swollen by moist heat. HOWARD and CURRIE (**17, 18**) extended the observations of LISTER and others to include the mycelia and fruiting bodies of a number of fungi. The several workers speak of the destruction of mycelial fungi by myxomycetes as parasitism, but perhaps such speedy and complete dissolution of the "host" should better be called preying.

CĚLAKOVSKÝ (**9**), extending the work of PFEFFER (**27**), studied the ingestion and digestion of living and non-living particles by plasmodia. He found that food vacuoles formed around particles which could be digested (egg white, starch, green algae, and vegetable cells), while generally those which could not be digested (fungus spores, living cells of *Euglena*, *Spirogyra*, etc.) were not surrounded by vacuoles. He found no evidence, in limited observations, of the digestion of the bacteria (*Bacillus megatherium* and *B. subtilis*), although the bacteria were surrounded by vacuoles. However, the ingestion and digestion of bacteria by swarm-spores of myxomycetes, if not by plasmodia, had been seen by LISTER (**22**).

REPORTS OF TWO-MEMBERED CULTURE.—That bacteria play a major role in the nutrition of the Acrasiales and therefore probably also in the nutrition of the myxomycetes, was shown by NADSON (**25**). NADSON was the first to claim pure culture of at least a myxomycete-like organism, *Dictyostelium mucoroides*. His bacteria-free cultures developed irregularly and finally died. Other cultures made in association with *Pseudomonas fluorescens liquefaciens* were normal. Although NADSON incorrectly considered the relation between

the bacteria and the *Dictyostelium* to be symbiotic (cf. RAPER, **30**) rather than two-membered,[2] his work indicates a careful technique, evinced by the rigid methods he employed in determining the purity of his cultures. NADSON showed that mere macroscopic examination of culture plates was completely unreliable, and that the real tests for purity depended on careful microscopic examination and on inoculations into media particularly favorable for the development of bacteria. We find no examples of equal care reported in the literature on the "true" myxomycetes until the work of VON STOSCH (**37**). For the further work on the Acrasiales, the reader is referred to RAPER'S (**31** *et prev.*) reviews and researches.

A definite indication that the true myxomycetes can derive a large part of their nourishment by the ingestion of cells is given by HENNEBERG (**14**). He fed yeast to the myxamebae and plasmodia of *Physarum leucophaeum* and was able to observe the intake, and often the subsequent ejection, of the yeast by the myxamebae. This egestion of cells after ingestion caused HENNEBERG to conclude that the slime sheaths or metabolic products secreted by the yeast were more important nutritionally than the cells themselves.

HENNEBERG'S paper was followed by a similar one by CHRZĄSZCZ (**10**), who interpreted the feeding in an altogether novel manner. According to CHRZĄSZCZ, the myxamebae and the yeast are in competition for the same medium, and the myxamebae attack and ingest the yeast cells merely to destroy them, the killed cells being later voided or perhaps digested for subsidiary nutrition.

That definite two-membered cultures of plasmodia and bacteria could be established was first proved by PINOY (**28, 29**). He sowed the spores of *Didymium difforme* and *D. effusum* on agar slants and found that some of the cultures remained altogether clear and without development of any sort. But those cultures in which bacteria also developed exhibited a vigorous growth of the plasmodia of these

[2] The term "two-membered culture" is used as a literal translation of the German *zweigliedrige Kultur* employed by protozoologists. *Zweigliedrige Kultur* has the advantage over such phrases as pure-mixed culture and association culture by being specific in meaning—the feeding of one living organism upon another which in turn thrives upon the supplied substrate.

"Pure culture" is employed here in its strict sense: a culture in which only one kind of living organism is present.

myxomycetes, and best results were obtained with *Bacillus luteus* as the second member of his two-membered cultures. PINOY concluded that the bacteria were used directly as food by the plasmodia.

Later VOUK (**39**) cultivated *Didymium nigripes* for 2 years in two-membered culture with a bacterium, probably *Pseudomonas fluorescens liquefaciens*. He was unable to obtain plasmodia in a state of purity and doubted whether it were possible to grow them so. In agreement with other workers, he found that media which allowed too rich growth of the bacteria were unfavorable for the cultivation of plasmodia. VOUK made an important technical contribution which seems to have escaped the notice of succeeding researchers; namely, that bacteria which accompanied the plasmodia formed neither well defined nor even diffuse colonies on the agar, but always were to be found in numbers at the margins of the plasmodial strands, at the periphery of the plasmodial front, and in the etched tracks left by the plasmodium as it crawled over the agar.

In 1928 SKUPIENSKI (**34**) reported the two-membered culture of *Didymium difforme* with *Aspergillus glaucus*, *Torula glutinis*, *Sarcina lutea*, *Bacillus prodigiosus*, and particularly vigorous cultures with a bacterium resembling *B. vulgaris*. SKUPIENSKI found no growth of the plasmodia in absolutely pure culture, and concluded that microorganisms were necessary for growth of this myxomycete. SKUPIENSKI had earlier (**32**) claimed the pure culture of *Didymium nigripes*.

In his work with *D. nigripes* SKUPIENSKI sowed the spores on the walls of tubes containing agar slants. The small plasmodia formed from the germinated spores crawled down from the glass to the agar, presumably leaving the bacteria behind. Normal growth and fructification occurred on the clear agar, this clarity apparently being accepted as complete proof of non-contamination.

VON STOSCH (**37**) cultivated plasmodia of several species of myxomycetes, especially *Didymium nigripes*, in two-membered cultures with *Torula* sp., beer yeast, an unknown mold, and several species of bacteria. After several transfers the vitality of the plasmodia declined, with the exception of *D. nigripes* in two-membered culture with a yellow micrococcus. Even here VON STOSCH believed the bacterial colonies to be composed of two species, not separable on hay

agar plates. Only negative results were obtained with attempted pure cultures on bacteria killed with heat or chloroform.

Finally WATANABE (**40, 41**) tested the ability of a wide selection of plasmodial species to grow on various bacteria and yeasts. Since the micro-organisms were streaked on plain washed agar, this is the first clean-cut case of the ingestion and assimilation of micro-organisms in the absence of other supposititious dissolved nutrients. Unfortunately WATANABE does not mention whether he grew the plasmodia through several serial transfers, and therefore the possibility of continued two-membered cultures of his plasmodia is left in doubt.

REPORTS OF PURE CULTURE.—Several workers (CONSTANTINEANU, SKUPIENSKI, CAYLEY, *et al.*) have grown myxomycetes in solutions or agars in which no particulate matter was intentionally supplied. A misinterpretation of their results has occurred in the literature; namely, that plasmodia can grow on dissolved nutrients alone. Indeed MACBRIDE and MARTIN (**23,** p. 7) state: "Several species have been grown in pure culture; among others, *Didymium difforme* (SKUPIENSKI, 1926, 1927, 1928) and *Didymium nigripes* (CAYLEY, 1929)." Yet on examination, the papers of SKUPIENSKI and CAYLEY give no basis for such a belief. For example, SKUPIENSKI says nothing concerning culture in his 1927 paper (**33**).

"Les spores sont toujours accompagnées par les bactéries qui se multiplient rapidement et servent de nourriture aux zoospores, myxamibes, et plasmodes" (**33,** p. 150).

"Cette espèce [*Didymium difforme*] vit en association symbiotique avec une Bactérie rapprochée de *Bacillus vulgaris*. Sans cette dernière son développement est très faible et n'arrive pas jusqu'au bout" (**34,** p. 327).

"... les spores germent au bout de quelques heures après leur ensemencement. Les bactéries, associées au Myxomycète, se développent avec la même intensité" (**35,** p. 205).

CAYLEY states: "No serious attempts have been made to eliminate bacteria from the cultures; in fact it is almost impossible" (**8,** p. 228).

The following abridged statement is from CONSTANTINEANU: "In Myxomyceten-Kulturen . . . die sich ansammelnden Stoffwechsel-

produkte teils von Bakterien, teils von Myxomyceten selbst herrühren, ohne dass es bisher möglich wäre, beide zu unterscheiden" (**12**, p. 525).

Of the two claims to pure culture made in the literature, SKUPIENSKI's has already been considered. HOWARD's contribution to the culture of the myxomycetes appeared in 1931. HOWARD's method (**16**) and CAMP's modification of it (**7**) made it possible to grow certain plasmodia in as great a quantity as desired. HOWARD makes no claim to pure culture, but states that after several transfers through non-nutrient plates the plasmodia "will be reasonably free of contaminating organisms and may then be transferred to oat agar."

In another paper (**15**) of the same year, HOWARD speaks of uncontaminated plasmodia growing well on plugs of sterilized *Polyporus frondosus*. However, the section of the paper called "food relations" leaves one in doubt about the purity of the cultures, owing to the seemingly contradictory statements made about the presence of other organisms.

From the foregoing review it is evident that there are only a few, probably doubtful, examples of true two-membered cultures (VOUK, PINOY, SKUPIENSKI, and VON STOSCH); and only two questionable instances of absolutely pure culture (SKUPIENSKI, HOWARD).

It seemed to the writer, therefore, that the controlled culture of these organisms should be studied in detail and adequate criteria of purity established. The results of the study are presented in this report.

Materials and methods

Most investigators used spore cultures to obtain uncontaminated plasmodia. The use of spores has the disadvantages of tediousness and of being limited to members of the genus *Didymium* and the few other myxomycetes which form plasmodia readily in culture. With the exception of *D. squamulosum*, therefore, which was regularly obtained from spore cultures, all the myxomycetes studied (*Badhamia foliicola*, *B. utricularis*, *Fuligo septica*, *Stemonitis axifera*, and the yet unidentified "plasmodium 51") were obtained as plasmodia or sclerotia in the field, and cultivated by a modification of HOWARD's

(16) method to insure a plentiful supply of material with which to attempt pure culture.

One of the two pure culture methods used by the writer was suggested by the work of WATANABE (40, 41) previously mentioned. Although the "enrichment" method of the writer is founded on WATANABE'S work, it was seen later that such two-membered cultures had been used for amebae by BEIJERINCK (4, 5) and thoroughly investigated as a method for the pure culture of amebae by OEHLER (26).

For purification itself, two methods were employed:

1. The migration method, in which plasmodia were allowed to crawl over several plates of non-nutrient agar.

2. The enrichment method, in which plasmodia were fed washed, living yeast cells in the form of a heavy suspension streaked on non-nutrient agar.

The writer has used and now suggests the following two criteria of pure culture for myxomycete cultures so obtained.

1. There must be no growth of micro-organisms in nutrient media inoculated from the cultures. The media should include at least one comparable in composition with the media used for pure culture of plasmodia, of approximately the same pH, and incubated at the same temperature for a sufficiently long time.

2. The cultures must be carried through several transfers without decline in vigor (13), and strictly should grow indefinitely on the pure culture media without a permanent loss of vigor.[3]

When employment of these criteria showed a plasmodium to be pure, it was inoculated into oatmeal agar simultaneously with a pure culture yeast suspension for the two-membered cultures; and into non-nutrient agar streaked with autoclaved bakers' yeast for the pure cultures.

The following media were employed, and are referred to by the assigned symbols in the detailed accounts of the outlined methods.

[3] Under conditions where factors such as temperature, humidity, and size of the inoculum are not readily controlled, it may be expected that cultures will show temporary changes in vigor. In the writer's cultures fluctuations are exhibited by *Physarum polycephalum* particularly, and by two-membered cultures in which the yeast grows faster than the plasmodium.

Media[4]

YEAST-EXTRACT DEXTROSE BROTH (YED)

Yeast extract (Difco, powdered)	2.5 gm.
Dextrose	10.0
$Na_2HPO_4 \cdot 12H_2O$ (Merck's c.p.)	1.4
Tap water	1 liter

This broth, dispensed into tubes, is used to test for contaminants in cultures. For growing the yeast utilized in the experiments the broth is modified by leaving out the disodium phosphate. Agar (2 per cent) is added for plates and for stock pure-culture slants of sugar-requiring micro-organisms.

YEAST-EXTRACT BROTH (YE)

This is similar to the preceding but without phosphate or dextrose. It is used in testing for contaminants in cultures and with 2 per cent agar for growing stock cultures of the bacteria employed.

AUTOCLAVED YEAST (YA)

Equal quantities by weight of bakers' yeast and distilled water are thoroughly mixed in a mortar and dispensed into tubes in 5-cc. quantities for autoclaving and storage. Before use, the tubes are gently agitated to suspend the yeast evenly. This medium is used for the pure culture of myxomycete plasmodia.

LIVING YEAST (YL)

The yeast is cultured in liter flasks, each containing 200 cc. of YED without phosphate, and the flasks are occasionally shaken for aeration. At the end of 48 hours the flasks are inclined until the yeast settles, and the excess medium decanted off through a flame. Sterile distilled water (about 50 cc. per flask) is added, the flasks well shaken, allowed to stand until the yeast again settles, and the washing repeated twice more. The final suspension is centrifuged and only a volume of water equal to the volume of the yeast layer is left. The tubes are stored until needed, preferably not longer than a week, at 6° C. This yeast is streaked on sterile WA plates of pH 6.0 for enrichment plates.

[4] All media are sterilized by autoclaving 20 minutes at 15 pounds' pressure, unless otherwise indicated.

The technique described is for *Saccharomyces ellipsoideus* strain 8.2.1 C.B.S. (*Centralbureau voor Schimmelcultures*). In all cases where C.B.S. cultures are mentioned the strain numbers are those of the Hopkins Marine Station from which the writer obtained all his C.B.S. strains. For very slimy or slow-growing yeasts, the technique is modified by longer period of incubation, solid substrates, heavier centrifuging, etc.

UNWASHED AGAR OF PH 6.0 (6.0 UA)

Agar (Difco Bacto, granulated)	20 gm.
$Na_2HPO_4 \cdot 12H_2O$	0.96
KH_2PO_4	1.45
Distilled water	1 liter

This agar has a pH stable near 6.0 even after repeated melting and pouring. The molar ratio KH_2PO_4/Na_2HPO_4 is 8/2, and the molar quantity is M/75 total PO_4. Slants of this agar, streaked with YA, are used for growing pure cultures of myxomycetes.

WASHED AGAR (WA)

One hundred gm. of granulated Difco Bacto agar are washed for 5 days with frequent changes of distilled water. After the last wash, as much water is decanted off as possible and the agar placed in a fine cheesecloth which is squeezed until all free water has been driven out. The pearly white, friable mass is weighed and stored in the refrigerator until needed. With 100 gm. dry agar the final weight should be between 500 and 600 gm. Two per cent agar (dry weight basis) is prepared and buffered in the same manner as the 6.0 UA. Because of the large amount of water it is best to subtract the weight of the washed agar from that of a liter of water so that the final weight is 1000 gm.

For any required pH, sample flasks are made up with 2 per cent agar and buffered by different ratios of Na_2HPO_4/KH_2PO_4, the total added phosphate always being M/75 in the final preparation, pH's of the samples taken after autoclaving (pH's may be changed by autoclaving), a curve of pH against KH_2PO_4/Na_2HPO_4 drawn, and agars of proper pH prepared by using the values interpolated on the curve.

Washed agar is used for migration and enrichment plates since unwashed agar has enough organic substance to support the growth of contaminants (fig. 2*C*).

Oatmeal Agar (OA)

Quaker Rolled Oats is dispensed into 125 ml. Erlenmeyer flasks to an even layer one grain in thickness, and 20 cc. 6.0 UA is poured into each flask. Just sufficient oatmeal is used to allow some of the grains to reach the surface after autoclaving (dispensing oatmeal agar into flasks was found to be unsatisfactory owing to the settling of grains while the agar was poured). This OA is used for stock two-membered cultures of plasmodia.

Glassware.—In these experiments only two kinds of special glassware are employed, the small calibrated pipettes and the vial-capped centrifuge tubes, and they are sterilized in dry heat by the usual bacteriological procedure.

The small pipettes for streaking the autoclaved and living yeast are prepared by drawing out 30-cm. lengths of 5-mm. exterior diameter soft glass tubing in the middle, and breaking. Best results in streaking are obtained with long slender nozzles, the tips of which are 1–1.5 mm. exterior diameter. These small pipettes are calibrated by marking at every centimeter with acid-and-heat-resistant paint (each centimeter corresponds to 0.05 cc.). They are subsequently sterilized in large, plugged test tubes.

Small (13×100 mm.), rimless, pyrex culture tubes, capped with inverted shell vials, are used for aseptic centrifuging of yeast cultures. The use of shell vials instead of cotton plugs avoids the objectionable accumulation of lint in the centrifugate.

Collection and gross culture.—Collected plasmodia are generally heavily contaminated and rather small. Preliminary to purifying, therefore, each plasmodium was partially cleansed of contaminants by allowing it to crawl over one or two plates of 2 per cent agar before attempts were made at culture. A modification of Howard's (**16**) oatmeal agar method was used for culture.

Paper toweling or filter paper was spread over the bottom of a moist chamber, wetted, the excess water allowed to drain off, and the plasmodium-bearing substrate placed at one side of the dish. As the

plasmodium crept over the paper, oatmeal was sprinkled on or near it, with especial care at first that the grains were spread neither too widely nor too thickly lest contaminants overgrow the culture.

For the steps preliminary to purification, a piece of paper toweling bearing the plasmodium was placed on a sterile agar plate to which the myxomycete soon migrated. Transfers were made from the agar to the media used in purification, and the rest of the plasmodium saved as a sclerotium by slow drying (**16**).

PURIFICATION METHODS.—In the first or migration method small pieces (approximately 2×1 mm.) of a plasmodium prepared as previously described were cut out, together with the underlying agar, and each placed in a 6.0 WA plate 1 cm. from the periphery. After the plasmodium had migrated over the plate as far as it could without crossing its own track, it was inoculated into a second similar plate and a small fragment inoculated simultaneously into a YED tube.

Three successive transfers of the myxomycete were generally sufficient to free a plasmodium of contaminants, and at each transfer a portion was placed in a YED tube which was incubated for 5 days at 25° C. before judgment was made on the purity of the plasmodium. Only rarely did a tube remaining clear for 5 days become cloudy later.

A pure plasmodium was inoculated into OA flasks simultaneously with the organism desired for two-membered culture, and into 6.0 WA slants streaked with YA for pure culture.

In the second, or enrichment culture method, each 6.0 WA plate was streaked along a diameter to a length of 5 cm. with a suspension of *S. ellipsoideus* (YL), the plasmodium placed at one end, and the plates examined daily for migration and feeding. After reaching the end of the streak the plasmodium was inoculated into a second similar plate and small fragments inoculated both into YE and YED tubes, which were incubated as described for the migration method. Both broths were used here, for although the YED is the more general culture medium, a heavy yeast growth often obscured slow-growing bacteria; YE served as a check since the yeast did not grow in it appreciably. On the other hand, a sugar-requiring contaminant would not grow in the YE and therefore might not be revealed.

After use of the enrichment method it was seldom necessary to inoculate *S. ellipsoideus* into two-membered cultures as the myxomycete usually carries sufficient yeast with it. But for pure cultures the plasmodium is desired without the yeast, and the *Saccharomyces* was removed by one or two migrations over non-nutrient agar.

PURE AND TWO-MEMBERED CULTURE.—Two-membered cultures were grown as described, in 125-ml. OA flasks into which the yeast and the plasmodium were inoculated simultaneously. Such cultures may thrive for a month or more without the need of transferring, but care must be taken that plasmodia are transferred before they decline too far in vigor, otherwise they may die overnight, presumably from the effects of accumulated wastes.

Attempts at using media other than oatmeal for cultures have not proved satisfactory, usually because these media, Pablum agar, YD agar, malt agar, and oatmeal mash, were richer and allowed too heavy a growth of the yeast.

Two-membered cultures have been attempted with organisms other than *Saccharomyces ellipsoideus*, such as *Torula aclotiana* (fig. 4*A*), the mycelia of *Alternaria* sp. and *Coprinus* sp., and various bacteria, but generally such cultures have not proved satisfactory.

For pure culture, slants of 6.0 UA were streaked with the autoclaved yeast in a manner exactly like that used for streaking living yeast. With the rough calibrations on the pipette as a guide, about 0.1 cc. YA was streaked the entire length of each slant and an uncontaminated plasmodium inoculated near the bottom.

Pure cultures were usually incubated at 25° C. to increase the rate of metabolism and hence the number of serial transfers in a given time. All other cultures were kept at room temperature (22° C.), although gross, two-membered, and pure cultures thrive between the temperatures of 12° and 28°, the limits used by the writer.

STORAGE OF CULTURES.—Gross cultures were sclerotized by the method of HOWARD (**16**) and kept in envelopes in the refrigerator. It is not yet known whether refrigeration adds to the length of time sclerotia may be kept in viable condition.

No altogether satisfactory means of storing two-membered and pure cultures could be devised. Pure cultures were allowed to sclero-

tize, while two-membered cultures were placed in the refrigerator at 6° C.

Pure culture sclerotia of *Badhamia foliicola*, the myxomycete most frequently used in these investigations, seem to remain viable for hardly more than a month. Only one out of about twenty-five germinated after 3 months, although most of the others were apparently

TABLE 1

PLASMODIA IN PURE CULTURE

PLASMODIUM	STRAIN NO.	NO. OF PASSAGES IN CULTURE	LOCALITY AND DATE COLLECTED
Badhamia foliicola List	13	15	Stanford University, California; oak leaves; April, 1936
Didymium squamulosum Fr.	14	9	Stanford University; bedding straw (as fruit); December, 1936
Fuligo septica Web.	15	14	Bakersfield, California; old willow logs; July, 1936
Stemonitis axifera MacBr.	21	3	Stanford University; preying on Auricularia sp.; December, 1936
Badhamia utricularis Berk.	22	4	Stanford University; preying on bracket fungus; January, 1937
Physarum polycephalum Schw.	24	6	Howard's original isolate, through courtesy Dr. William Seifriz
Plasmodium 51 (has not fruited)	51	8	Cambridge, Mass.; preying on Exidia on dead elm branch; April, 1938

normal under the microscope and did not discharge their color on wetting—a property of dead or moribund plasmodia and sclerotia. The use of infusion agars of various natural substrates (hay, leaves, etc.) was unsuccessful, as were 0.02 molar concentrations of the excystment substances citrate and malate (**38**). Sclerotia of two-membered and gross cultures of the same species are viable for a longer period, gross culture sclerotia having been brought into a vegetative state after 2 years of dormancy.

The species listed in table 1 have been obtained in pure and two-membered cultures by one or both of these methods.

Results

Purification experiments

It is apparent that migration over non-nutrient agar might eventually free a plasmodium of contaminants by simple attrition and by the washing of the protoplasmic surface by the constantly secreted slime. Indeed it has been observed that plasmodia with an adherent tenacious slime (*Fuligo, Didymium*) are less easily obtained pure by migration than are those with a very loose, abundant slime (*Physarum, Badhamia foliicola*). It was therefore surprising that the first attempts at obtaining uncontaminated plasmodia by migration gave completely negative results, so the method was abandoned in favor of enrichment.

An enrichment culture meets the minimum requirements of the organism desired in mass culture, but at the same time it does not satisfy the needs of the associated undesired organisms. Living, washed yeast cells can serve as food for only those organisms which can engulf them, and therefore are admirable for plasmodial enrichment cultures.

For the early specific enrichment cultures several yeasts were employed. Of these, *Saccharomyces ellipsoideus* Hansen, 8.2.1. C.B.S.; *S. cerevisae* Hansen, 8.1.6. C.B.S.; and FF-17 Department of Bacteriology, Stanford University, gave the most promising results. The strain of *S. ellipsoideus* was found to be the most useful yeast as it is easily grown in liquid culture under not too critical conditions, is quickly eliminated by plasmodia crawling over plain agar as shown in figure 2*B*, and in addition could be used to grow all plasmodia on enrichment plates (with the exception of *Physarum polycephalum*) which could be grown on oatmeal agar. The *S. cerevisae* strains were almost equally useful, but being bottom yeasts their tendency to clump together and settle out quickly hindered even streaking.

It may be noted that although other yeasts used[5] often were

[5] *Debaryomyces hudeloi* da Fonseca, M-182; *Nectaromyces alpinus* Kluyver, M-1; *Willia anomala* Hansen, M-63; *Zygosaccharomyces pastori* Guill., M-174; all of the Harvard Medical Collection, and *Sporobolomyces salmonicolor* Kluyver & van Niel, 28.1.1 C.B.S.; *Torula aclotiana* Kufferath, 15.16.1 C.B.S.; *T. dattila* Kluyver, 15.1.1. C.B.S.; *T. monosa* Kluyver, 15.2.1. C.B.S.

favorable for growth of the plasmodium, they were impracticable because they were slow-growing in culture, or were small, glutinous, and therefore very difficult to remove from the plasmodium—disadvantages lacking in *S. ellipsoideus*.

After the plasmodia have been shown to be free of contaminating organisms, enrichment can often be carried a step further to rid the myxomycete of its yeast; all that is required is a sugar-free medium which supports the growth of myxomycetes. Such a medium is sugar-free autoclaved yeast. But since most commercial brands of bakers' yeast contain appreciable amounts of sugar after autoclaving, it has been found preferable to allow plasmodia from enrichment plates to migrate over one or two plates of pH 6.0 washed agar before placing them on autoclaved yeast slants which contain enough sugar to allow the growth of *S. ellipsoideus*.

At first considerable success was obtained with the enrichment method, but later plasmodia (*Fuligo septica*, *Didymium squamulosum*) were obtained in the field which resisted nearly all attempts to free them of contaminants. These plasmodia fed well but slowly on the yeast, and often arrived at the end of streaks more visibly contaminated than when they were inoculated. Sometimes a veritable garden of pseudomonads and bacilli could be observed at the advancing plasmodial edge under the microscope. From those amenable to the enrichment method these plasmodia differed in forming long straggling masses often extending back to the point of inoculation, and in their slow feeding especially (fig. 1*A*, *B*). Under these conditions the yeast, always in a state of inanition on washed agar plates, died and autolyzed, thereby furnishing nutrient for the contaminants and destroying the conditions of enrichment. Yeast cells removed from these streaks and examined in weak aqueous methylene blue, which stains only dead cells, showed many cells, even up to half in the field, staining with the dye, while yeast removed from a culture of a relatively fast-moving plasmodium showed very few cells taking the stain after the plasmodia had crawled comparable distances.

These plasmodia so refractory to the enrichment method were obtained in pure culture by a modification of the formerly unsuccessful migration method. In the earlier method, large (with a fan 0.5 cm.

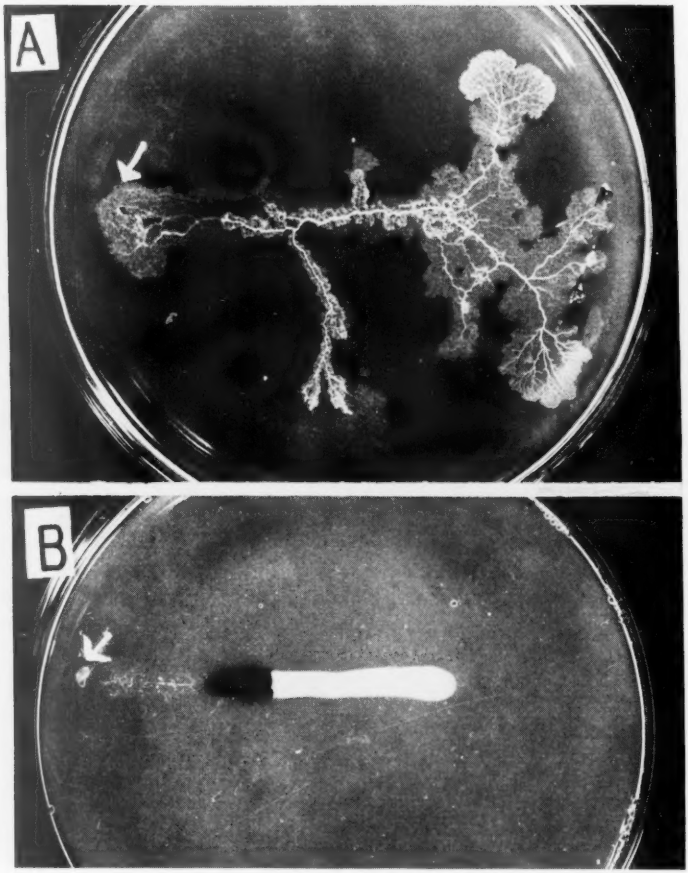

FIG. 1.—Types of feeding exhibited by plasmodia on living yeast enrichment plates (YL, 6.0 WA). *A: Fuligo septica*, slow-feeding plasmodium with diffuse fan. *B: Badhamia foliicola*, fast-feeding plasmodium with compact fan. With orthochromatic plates these bright orange-yellow plasmodia photograph as black (*cf.* fig. 4*A* and compare fig. 4*B*, taken with panchromatic plate). White streak extending to right of plasmodium is the yeast not yet consumed. Arrows in *A* and *B* point to sites of inoculation.

diameter or larger) plasmodia were allowed to migrate, while in the later successful method smaller plasmodia were employed.

The impression that the rate of feeding determined comparative usefulness of the enrichment and migration plate methods was checked more critically by measuring the rates of crawling on both kinds of plates. At the same time the possible influence of pH on crawling rates was observed.

In these studies washed agar plates at pH 5.5 and at pH 6.5 were prepared and half the plates at each pH streaked with a suspension of YL for a length of 5 cm. along the diameter of each plate. Fragments from contaminated plasmodia were placed on each migration and enrichment plate and the cultures incubated in the dark at room temperature (22° C.). For uniformity, the inocula were of as nearly the same size as possible and were taken from similar regions of each plasmodium. Because of the diminution of size on inanition, the inoculations for the migration plates were larger (0.5 $cm.^2$) than those for the enrichment plates (2 $mm.^2$). Plates were examined daily and distances migrated on both kinds of plates noted. After a migration of 5 cm., plasmodia were transferred to corresponding plates and at the same time inoculated into broths. Three such serial plates were used for each culture.

Each migration rate recorded in table 2 is an average of eight plasmodia of the same clone. Migration rates of the starred (*) plasmodia included in the table were measured previous to the rates of the others. Hence the data though obtained under similar conditions are not strictly comparable, but are included because of their interest.

It is seen from table 2 that for the enrichment method to be feasible, plasmodia must crawl up the streak at a minimum rate of 1.2 cm. per 24 hours. A comparison of the number started, number pure, and number of plasmodia remaining at the conclusion of the experiment shows that not all plates in a series could be carried through to completion. Thus *Fuligo septica*, plasmodium 52, often sclerotized on the migration plates and could not be revived, a tendency not exhibited to so marked a degree by other strains of this species.

The extremely fast-moving plasmodia *Badhamia foliicola* and

Physarum polycephalum were in nearly all cases pure at the end of the first passage on the migration plates. *P. polycephalum* spread so rapidly that its rate could not be measured by daily inspection, and therefore it is indicated only as more than 5 cm. per day.

TABLE 2

Plasmodium	pH of agar	Migration over yeast streaks				Migration over plain agar			
		Daily crawling rate (cm.)	No. of cases started	Total pure at end	Total at end	Daily crawling rate (cm.)	No. of cases started	Total pure at end	Total at end
Badhamia foliicola	5.5	1.4	8	8	8	6.2	8	8	8 (1st passage)
	6.5	1.2	8	8	8	6.8	8	7	8 (1st passage)
Physarum polycephalum	5.5	No feeding in any case			.....	5+	8	8	8 (1st passage)
	6.5	No feeding in any case			.....	5+	8	8	8 (1st passage)
Stemonitis axifera	5.5	1.6	8	6	7	4.1	8	8	
	6.5	1.8	8	3	4	4.8	8	8	8
									8
Plasmodium no. 51	5.5	1.7	8	5	6	2.3	8	1	2
	6.5	1.4	8	5	6	3.1	8	3	4
Fuligo septica no. 52	5.5	0.6	8	0	3	2.0	8	0	1
	6.5	0.6	8	0	5	1.9	8	1	2
Fuligo septica* no. 15	5.0–7.0	1.6	20	9	19	1.5	20	19	20
Didymium squamulosum*	6.0	1.0	6	0	6	.....	6	6	6

* See statement in text.

The behavior of *Fuligo septica* (plasmodium 15) in this particular experiment is an apparent exception to the slow-moving *Fuligo* spp. Neither before nor after this experiment has any success been obtained with the enrichment method with plasmodium 15 nor any other *Fuligo*.

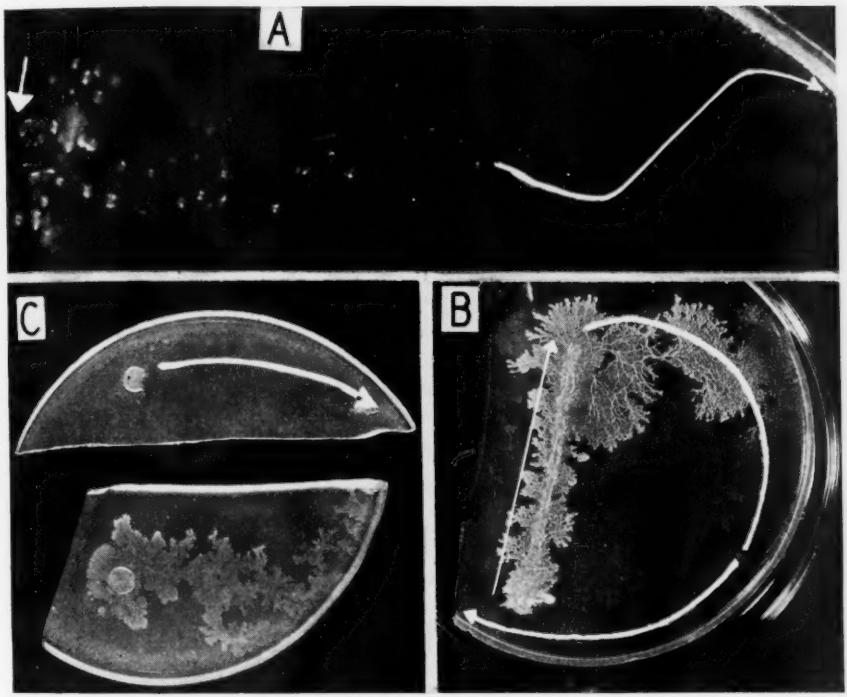

FIG. 2.—Growth of micro-organisms left in situ by plasmodia which in all cases have been removed and their paths indicated by long curved arrows. In *A* and *B*, agar was placed on surface of nutrient plates; the nutrient diffusing upward allowed growth of the deposited micro-organisms into colonies. *A:* migration plate showing rapid loss of bacteria indicated by thinning out of colonies with increasing distance from inoculum (indicated by short arrow). *B:* enrichment plate showing speedy loss of *S. ellipsoideus*. Slender arrow indicates extent of yeast streak. Break in heavy arrow shows last yeast colony developing. Note extent to which plasmodium has spread the yeast to form an image of itself. *C:* differences in nutrient content of washed and unwashed agars. Upper portion, from washed-agar migration plate, shows no development of bacteria in track of plasmodium. Lower portion, from unwashed-agar plate, shows development of bacteria without addition of nutrient.

It is also seen from table 2 that there was little difference in the obtained results ascribable to the influence of the hydrogen ion concentrations in the range employed. The agar becomes increasingly soft and difficult to work with as the pH decreases, but on the other hand, agars of pH's higher than 6.5 become increasingly unfavorable to the vigor of the myxomycete. Agar of pH 6.0 lacks these disadvantages and was therefore employed in all subsequent work.

The migration method, besides being simpler, gives apparently better results on the whole than does the enrichment method. This superiority is only apparent, for it has too often happened that a plasmodium has been so completely devitalized by continued inanition that it could not be transferred successfully to two-membered or pure culture. The enrichment method when feasible always insures an abundance of plasmodium with which to work; there is no danger of sclerotization, and little danger of the plasmodium crossing its own track and picking up contaminants it had left behind.

The freeing from bacteria was illustrated more qualitatively and picturesquely by cutting out the non-nutrient agar from an enrichment or migration plate over which a plasmodium had crawled and placing it on a yeast-extract-dextrose agar plate. The nutrient diffused up through the plain agar and allowed development of colonies from the deposited micro-organisms *in situ* (fig. 2*A*). The slowly moving plasmodia do not rid themselves very speedily of bacteria, and indeed the number of bacteria on the agar may be so great that the colonies coalesce.

By the same means the rapid loss of *S. ellipsoideus* could be demonstrated as shown in figure 2*B*, in which the last visible colony developed when the myxomycete had migrated about 7 cm. after leaving the end of the depleted yeast streak.

Adaptation phenomena

It is well known among protozoologists and bacteriologists (**20**, p. 162 *et seq.*) that organisms of a species transferred to a new medium may show extremely wide variation in growth in parallel cultures, and also that organisms may be trained to grow on a medium previously unsuitable. A similar phenomenon has been observed by the writer in the culture of myxomycetes.

In the course of these experiments it was found advantageous to make several parallel inoculations of a newly purified plasmodium into the slants of autoclaved yeast. Of several such cultures started, often only a few began development, and from the most robust of these (fig. 3*B*, *C*) the myxomycete was serially subcultured. Alternatively, fewer tubes were used and a very large inoculum placed in each (as in BRAUN and CAHN-BRONNER'S technique for the adaptive nutrition of bacteria, KNIGHT *loc. cit.*), but this mass-inoculation method is technically inferior, for the smaller the inoculum in a successful subculture on a new medium the more assurance there is that the medium is complete (**13**).

From the few experiments so far carried out, three adaptive phenomena may be recognized:

1. A plasmodium may be trained to grow under conditions which are usually inimical. For example, *Badhamia foliicola* was made to grow at a pH of 7.4 by both direct mass inoculation from a milieu of pH 6.0 and by using small inoculations and gradually raising the pH at each transfer. Small inoculations made directly into an identical medium remained immobile and died.

2. A plasmodium may be trained to utilize an initially poor nutrient. For example, *Physarum polycephalum* refused to grow in two-membered culture on oatmeal with *S. ellipsoideus* and in pure culture on autoclaved yeast when first inoculated in the usual manner. By the use of a number of inoculations in the first case, and of mass inoculation in the second, successful cultures were obtained. The writer has observed a higher degree of variation of behavior with *P. polycephalum* than with any other plasmodium.

3. A plasmodium regularly increases in vigor of growth with serial transfer on a new medium, and often within the first transfer (fig. 3*A*). This increasingly efficient utilization of the medium is not due to the size of the inoculum, for equally small inocula take up the yeast completely from the first on subculture. With the exception of the *Fuligo* spp., the writer's plasmodia all show OEHLER'S (**26**) *wallartig* instead of his *zerstreute Wachstum*.

Since all the plasmodia studied showed one or more of these adaptation phenomena, it seemed doubtful at first whether controlled nutritional work could ever be done with such variable or-

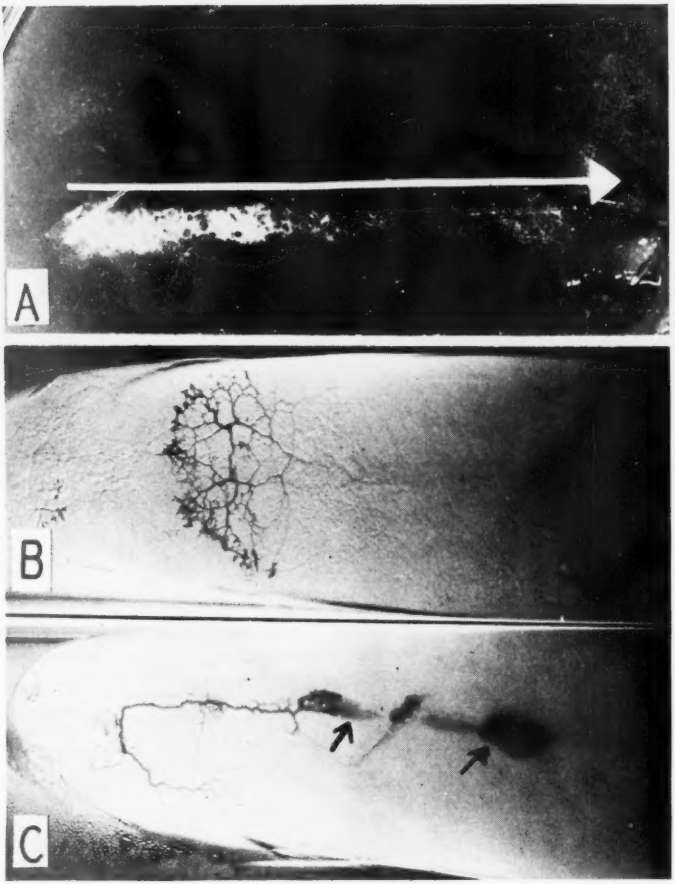

FIG. 3.—Adaptation phenomena exhibited by myxomycete plasmodia. *A:* increased utilization of *S. ellipsoideus* by plasmodium (plasmodium 51) on enrichment plate. Plasmodium inoculated at point indicated by tail of arrow leaves decreasing amounts of yeast behind as it migrates to position indicated by arrowhead. *B, C:* plasmodia of *Badhamia foliicola* from same clone in which differences of vigor in growth exhibited by original isolates have persisted through second transfer. *B:* second transfer on autoclaved yeast from isolate showing good growth on this medium. All yeast has been utilized and plasmodium is now in state of inanition. *C:* second transfer from isolate showing poor growth; same time interval as for *B*. Note large masses of untouched yeast (indicated by arrows).

ganisms; but continued cultures have produced laboratory strains which are highly consistent in their behavior under similar conditions.

Factors influencing culture

The inadvisability of a rich medium which allows excessive growth of the second member has already been mentioned in connection with two-membered cultures. In pure culture work it was soon observed that too much autoclaved yeast was inimical or even fatal to growth. There seemed to be two possible ways of interpreting this phenomenon: (*a*) too much dissolved organic material was present; (*b*) too heavy a layer of yeast physically hindered feeding of the plasmodia. Experiments undertaken with *Badhamia foliicola* indicated physical hindrance to be the factor in this instance.

In the experiments, two sets of autoclaved yeast were prepared. In both the yeast was autoclaved in the usual manner, but in one set soluble substances were washed away by repeated centrifuging in distilled water and the yeast re-autoclaved after adjusting the suspension to initial cell density, while the second set was merely re-autoclaved. The following series of media were prepared:

1. Slants containing 8 cc. of 2 per cent unwashed agar, pH 6.0, containing 1 cc. of the supernatant fluid of autoclaved yeast, and each streaked with 0.1 cc. of a suspension of washed, re-autoclaved yeast cells.

2. Slants containing 8 cc. of agar prepared as before, but each streaked with 0.1 cc. of a suspension of unwashed, re-autoclaved yeast cells.

3. Slants containing 8 cc. of 6.0 unwashed agar each streaked with 1 cc. washed, re-autoclaved suspension.

4. Slants containing 8 cc. of 6.0 unwashed agar each streaked with 1 cc. unwashed, re-autoclaved yeast suspension.

Only series 1 and 2 gave good growth, and therefore it seems clear that it is the quantity of yeast cells on the surface rather than the soluble substances which are inhibitory.

At one stage of the research, maintenance of all myxomycetes in culture was attempted on washed agar in order to reduce the number of separate media and the number of unknown factors contained in

unwashed agar. These cultures without exception decreased in vigor on passage until finally some gave no perceptible growth after transfer. It seemed obvious that some necessary substances, probably minerals, had been leached out by the washing process; and as plasmodia grew well in enrichment cultures on washed agar, these presumable substances must be in intact yeast cells and in unwashed agar. That unknown minerals are necessary was shown by the following experiment.

The ash of unwashed agar was added to washed agar (2 per cent) adjusted to a final pH of 6.0, slanted, and streaked with autoclaved yeast. Controls of unwashed agar and washed agar without minerals were run, and *Badhamia foliicola* was used as the test myxomycete because of its sensitivity to the washing process. Growth on the washed agar was much less than on both the unwashed agar and the washed-agar-plus-agar-ash, little if any difference between the latter two being noticeable.

Attempted pure culture on other media

Various investigators have used a number of media for culture of the myxomycetes. In most instances the culture was admittedly or obviously gross, and need be discussed no further here (DeBary, Lister, Klebs, Constantineanu, Miller, Belař, *et al.*).

Early in the present work it was expected that myxomycetes would grow well on pure culture on oatmeal agar (**16**), and that they would also grow on other common vegetable substrata. Actually no such growth took place when a few flasks and tubes of oatmeal agar were inoculated with *Physarum polycephalum* freed of contaminants. In a similar series of experiments with *Badhamia foliicola*, plugs of autoclaved potato, carrot, banana, and apple were also employed with negative results. No growth of either plasmodium was found in attempted pure culture on the following autoclaved media: yeast extract agar, yeast extract-dextrose agar, malt agar, oatmeal agar, oatmeal mash, pea mash, pea agar, pea infusion agar, *Agaricus campestris* sporophores, and *A. campestris* sporophore agar. That some of these media were not particularly inimical to development was shown by the growth of plasmodia in accidentally contaminated

vessels. In short, wherever bacterial contamination occurred, growth took place.

Because of the importance which oatmeal has achieved as a substrate for the cultivation of plasmodia (**15, 16, 7**) and the belief that it is a pure culture and a "synthetic" medium (**36**), it was more thoroughly investigated as a possible medium for pure culture and as a medium for two-membered culture.

In the earlier experiments three factors besides the lack of microorganisms may have been responsible for the failure of the plasmodia to grow: (*a*) the oatmeal grains imbedded in the agar may have been physically unavailable; (*b*) the inocula may have been too small; and (*c*) the hydrogen ion concentration may have been unfavorable.

To settle the first and third points, oatmeal agar of pH 6.0 (a pH known to be favorable) was prepared in the ordinary way, and for comparison, flasks of 6.0 unwashed agar were sprinkled with sterile oatmeal grains after the agar had solidified. HOWARD (**15**) mentions the difficulty of freeing *Physarum polycephalum* from a small toruloid yeast. Using this fact as a clue, two-membered cultures of *Torula aclotiana* 15.16.1 C.B.S. and *P. polycephalum* were made on both the oatmeal agar and the sterile oatmeal grains. Large (even to an inoculum covering half the surface of the flask) (*cf.* fig. 4C) and small inoculations were made into the two media, each with and without the *Torula*. Abundant growth was obtained on both media wherever the *Torula* was present, while no growth was perceptible in its absence (fig. 4). It may be mentioned that *P. polycephalum* has shown consistently better growth on *T. aclotiana* than on any other organism in two-membered culture on the same supplied substrate.

This problem was investigated further. *Physarum polycephalum* and *Badhamia foliicola* were grown in gross culture for one week on oatmeal at the slightly low hydrogen ion concentration of pH 7.0 to favor growth of bacteria. The bacteria were isolated by streaking on oatmeal decoction agar and pure culture isolations made from the different colonies.

Two-membered cultures were prepared by inoculating pure culture plasmodia into the oatmeal agar and "oatmeal-grain" flasks and placing a few drops of a suspension of each bacterium isolated directly on the plasmodial inoculum. Controls were (*a*) pure culture

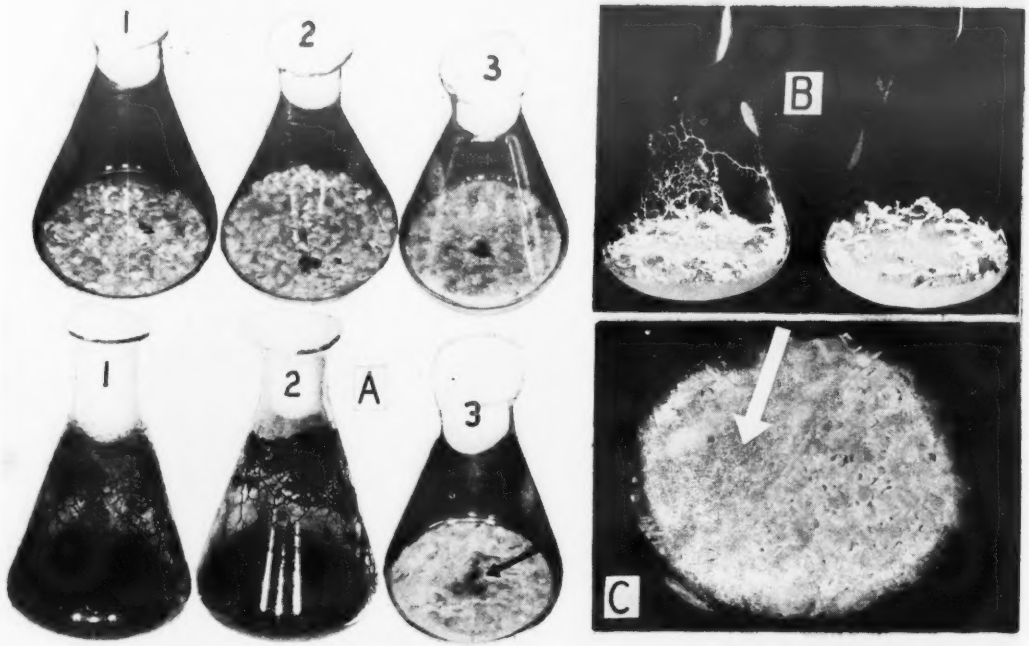

FIG. 4.—Two-membered and attempted pure culture on oatmeal. *A:* plasmodia of *Badhamia foliicola* on oatmeal agar, left to right (both rows): *1*, two-membered culture with *Torula aclotiana; 2*, same with *Flavobacterium ceramicola; 3*, attempted pure culture on oatmeal. Upper row, 12 hours after inoculation; lower row, 2 weeks later. Arrow in *3* points to dead inoculum in attempted pure culture flask. *B: Physarum polycephalum* with sterile oatmeal on agar. Left: two-membered culture with *T. aclotiana* showing excellent development of plasmodium. Right: attempted pure culture showing complete absence of growth. *C:* large inoculum of *P. polycephalum;* agar on which plasmodium was carried into flask covers left half of oatmeal agar surface. Plasmodium eroded exposed oatmeal agar at right of inoculum; in absence of second member it crawled back to original substrate and died after no perceptible growth. Arrow points to disintegrated plasmodium.

plasmodia inoculated into the flasks without bacteria, and (*b*) plasmodia inoculated into flasks with suspensions of scraping from gross cultures.

In most of the flasks inoculated with bacteria there was development, often to so great a degree that the agar was riddled with plasmodial strands, the walls of the flasks covered, and even the cotton plugs penetrated by the plasmodium (fig. 4*A*). In those flasks which contained no demonstrable living organism other than the plasmodia there was little or no development, the plasmodia crawling about sluggishly, eroding the grains only slightly, and finally breaking up into small pulvinate masses which died and discharged their color into the substrate. The controls inoculated with the mixture of organisms from gross culture showed some development, and *Physarum polycephalum* fruited in several of the control flasks; but generally the rank growth of molds, yeasts, and various bacteria inhibited the further development of plasmodia from reaching a degree commensurate with that of the most favorable two-membered cultures.

In those few two-membered cultures in which growth did not take place it could be shown that the bacteria raised the pH to an unfavorable degree, grew excessively fast, or formed slimy, mucilaginous colonies difficult to attack.[6]

Attempts have been made to obtain pure culture on oatmeal agar with the other myxomycetes in stock, but the results have been essentially the same as in the cases of *Physarum polycephalum* and *Badhamia foliicola;* namely, sluggish crawling, slight erosion of the grains, breaking up into small pulvinate masses, and finally death, after little or no perceptible increase in size of the plasmodium during its life on the oatmeal. *Fuligo septica*, to be sure, did show a very slight growth, but it too died within 3 weeks; in contrast this organism has been carried without transfer in regular two-membered culture for over 6 weeks at room temperature and over 3 months at 6°.

In these experiments no essential difference was found in the use of very small inocula and of those of 0.5 cm. or more in fan diameter.

[6] The relationships existing in two-membered cultures and the erosion of oatmeal grains even by pure culture plasmodia are to be taken up in a succeeding paper.

The smaller inocula took longer to grow to the same final quantity as the larger ones, and the conditions were somewhat more critical, as a fast-growing second member could overrun and stale the culture before the myxomycete became sufficiently established to keep it in check.

Discussion

Type of nutrition

That the plasmodia are not in competition with the yeast for the supplied medium, as Chrząszcz (**10**) stated, is shown by the experiments in which yeast alone supplied sufficient nourishment, and in its absence on a given medium there was no growth. Nor is there any indication that the secreted slime or metabolic products of the yeast are the important nutrients, as suggested by Henneberg (**14**). On the contrary the yeast cells are engulfed and destroyed, as microscopic examination of a yeast streak over which a plasmodium has crawled and fed reveals only a very few scattered cells instead of a densely packed, opaque yeast mass, or even the empty cell walls of such a mass.

Pure culture

Skupienski (**34**), von Stosch (**37**), and Pinoy (**28, 29**) declare it impossible to grow myxomycete plasmodia in pure culture, while Howard (**15**) and Skupienski (**32**) claim pure culture on media similar to those unsuccessfully employed by the writer (oatmeal agar, carrot agar, hay agar, and agaric agar).

These discrepancies may be explicable in the light of the present investigations. For example, it has previously been shown that for some plasmodia a number of transfers must be made to a pure culture medium before a few of the inoculations are successful. This was found to be particularly true of *Didymium squamulosum*, *Physarum polycephalum*, *Stemonitis axifera*, and *Fuligo septica*. So great is the variation with the freshly isolated *Didymium* that at present the writer is not certain that it can be carried through indefinitely in pure culture.

Other factors have been discussed, such as size of inoculum, amount of minerals present, and concentration of nutrients in the projected pure culture medium. In view of these complicating fac-

tors, it is perhaps not surprising that SKUPIENSKI (**34**) and VON STOSCH obtained negative results. The hydrogen ion concentration, although not very critical, may also have been left out of account.

SKUPIENSKI (**32**) claimed pure culture of *Didymium nigripes* on carrot infusion agar. HOWARD (**15, 16**) indicates the same results for *Physarum polycephalum* on oatmeal agar and sterilized fungus sporophores. The only criterion of purity recorded in the French résumé of SKUPIENSKI'S paper and by HOWARD (personal communication) is the clarity of the surface of the agar.

NADSON (**25**) long ago pointed out the unreliability of such a procedure, and VOUK (**39**) subsequently working with *D. nigripes*, the same species used by SKUPIENSKI, showed that plasmodia seldom leave well defined colonies in their wake but that the bacteria are distributed along the track and around the periphery of the plasmodium.

In consideration of the manner in which a plasmodium sweeps over a surface, it is difficult to expect contaminants to form defined colonies, and it is well known among microbiologists that the plating method requires a dry and undisturbed surface, especially in the case of very motile pseudomonads and those bacteria which form fluid mucilaginous colonies. Almost all the twenty-five bacteria isolated from the six different plasmodia produce on agar peculiarly nondescript and spreading colonies of a white, buff, or yellow color, while some colonies are practically transparent. In fact all the bacteria occurring commonly in the streak plates, and presumably the most abundant species in the gross cultures, could be so characterized.

When we add to the inherent difficulties just mentioned the opaque, heterogeneous appearance and light buff color of oatmeal agar, its low protein content, and the usually high acidity of such vegetable media, it is not surprising that bacteria which form such nondescript colonies should be difficult to detect macroscopically. When in addition bacterial colonies are broken up and spread either artificially or by the divagations of a restless plasmodium, only careful microscopical and cultural examination can be expected to reveal the presence of the contaminants. For example, uninoculated flasks have been compared with those inoculated over the entire surface

with an extremely actively growing pseudomonad common in all oatmeal gross cultures examined, and during the period of most active development only a slightly moister-appearing surface of the inoculated flasks (as if they were fresh from the autoclave) as compared with the controls indicated the presence of the pseudomonad, although a suspension prepared from the inoculated flask showed a field crowded with the actively motile bacteria. As a matter of fact even in vigorous two-membered culture, the ordinarily very visible and defined colonies of *Torula aclotiana* and *Saccharomyces ellipsoideus* are often spread out and overrun by the plasmodium to such a degree that microscopic examination is necessary to demonstrate the yeast.

It is pertinent at this point to quote from OEHLER's paper on a parallel research and discussion of protozoan cultures:

"Wie bei allen Bakterien-Amöbenzuchtversuchen, so ist auch bei der Amöbenzucht auf abgetöteten Bakterien ständige Prüfung auf Reinheit und Sterilität bei jedem einzelnen Versuchsakte nötig. Wenn man nicht immer und immer wieder alle Versuchsglieder auf Reinheit und Sterilität nachprüft, kann man das Wunderbarste finden" (**26,** p. 185).

Summary

1. The literature on the nutrition of myxomycete plasmodia is discussed critically from the standpoints of ingestion, two-membered culture (a special case of association culture with two different organisms), and pure culture. It is shown that the reports of pure culture are based on misconceptions or are at least open to criticism.

2. Techniques are described whereby myxomycete plasmodia may be obtained free of contaminants and subsequently grown in two-membered culture with *Saccharomyces ellipsoideus*, or some other micro-organism, and in pure culture on autoclaved yeast. Plasmodia may utilize living or autoclaved yeast as the sole source of nutrient.

3. The behavior of plasmodia in culture is discussed, and it is shown that myxomycete plasmodia may exhibit adaptation to cultural conditions.

4. Attempted pure culture on oatmeal and similar vegetable media was unsuccessful, in contradiction to reported pure culture on these media.

The writer is happy to acknowledge his gratitude for the many valuable suggestions and helpful criticisms of Dr. L. R. BLINKS and Dr. C. B. VAN NIEL who supervised the early stages of this work at Stanford University and at The Hopkins Marine Station of Stanford University, and to Dr. W. H. WESTON, JR. and Dr. K. V. THIMANN who directed the later work at Harvard University.

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USE OF THE FEULGEN REACTION IN CYTOLOGY
I. EFFECT OF FIXATIVES ON THE REACTION

B. B. HILLARY

(WITH THREE FIGURES)

Introduction

The Feulgen reaction was developed in 1924 by FEULGEN and ROSSENBECK (11) as a microchemical test for thymonucleic acid. This reaction was primarily intended to distinguish between the type of nucleic acid (thymonucleic) considered to contain a hexose radical and other similar acids containing pentose radicals. At the time FEULGEN carried out his experiments the exact nature of the carbohydrate complex of the thymonucleic acid molecule was not understood. Since then the investigations of LEVENE, MIKESKA, and MORI (17) have shown that the carbohydrate is not a hexose but a pentose. Notwithstanding this, the reasoning of FEULGEN and his collaborators regarding the theory of the reaction still holds.

The Feulgen reaction is carried out in two steps. The first is mild acid hydrolysis, which breaks the binding between the purine bases and the carbohydrate complex of the nucleic acid. This splitting off of the purines frees the aldehyde groups of the aldo-pentose sugars. The second step involves a chemical reaction between the liberated aldehyde groups and leuco basic fuchsin (fuchsin sulphurous acid). According to FEULGEN, this results in the synthesis of a new dye compound, which is violet in color as compared with the red of the basic dye.

It was formerly considered that there was a difference between the nucleic acid of plant and animal nuclei. Recently FEULGEN, BEHRENS, and MAHDIHASSAN (10) have developed a method of separating the nuclei of wheat germs from other parts of the cells and thereby extracting a pure plant nucleic acid from the nuclei. This nucleic acid was found to contain exactly the same groups as thymonucleic acid. Thus plant and animal nucleic acids are one and the same thing and the nucleal reaction works equally well with both.

FEULGEN (8) has demonstrated the value of the nucleal stain in both plant and animal materials. He has shown the great selectivity and specificity of the stain, in that it stains only the chromatin parts of the nucleus and leaves unstained the nucleolus as well as all parts of the cell outside the nucleus. This makes it very valuable for cytological work, as it stains the chromosomes with remarkable sharpness and clearness. Unlike most other cytological stains it does not require differentiation to make the chromosomes distinguishable; therefore it provides a much more certain and usually a much clearer picture of the finer details of chromosome structure.

In spite of these apparent advantages, adoption of the Feulgen nucleal stain by cytologists has been slow. With plants this is perhaps due to the fact that the reported differences between plant and animal nucleic acids made investigators feel that the reaction was uncertain in them. Another cause is the difficulty of obtaining a basic fuchsin suitable for use with the Feulgen reaction, as the impurities of the stain sometimes color the cytoplasm. The investigations of DE TOMASI (28) and SCANLAN and MELIN (23) have shown that this can be overcome by the use of a specially purified pararosanalin dye. COLEMAN (7) found a simple and more efficient method of overcoming this difficulty. By filtering the bleached solution with active charcoal, which removed the impurities, an absolutely colorless leuco basic fuchsin could be obtained from any sample of basic fuchsin.

Another retarding influence to the use of this stain was that FEULGEN recommended the use of a sublimate-acetic fixative, which is a poor cytological fixative. His reason for recommending this was that he considered fixatives containing oxidizing compounds such as chromic acid would be apt to destroy the nuclear material. Likewise he warned against the use of formalin because it is an aldehyde which would give the same color reaction. We now know that these compounds, at the concentrations used in fixatives, have no deleterious effect on the stain.

One of the greatest drawbacks to the use of this stain is the fact that there have been frequent and often incorrect reports of a negative stain reaction. For example, BOAS and BIECHELE (4) report a negative reaction in 138 out of 224 plants investigated. These results

cannot be accepted because the methods used were faulty. MILOVIDOV (**20**) claims that in many cases such results are due to the presence of tannins which inhibit penetration of reagents, but careful investigations which will be discussed later have shown that tannins do not normally inhibit the stain. *Spirogyra* was continually reported to give a negative reaction (**15, 16, 20, 22, 26**) until it was shown by GEITLER (**12**) that the stain was confined to widely separated minute chromocenters which had been overlooked by previous investigators. Such has been found to be the case in many forms.

In the extensive literature on the Feulgen reaction discussed by MILOVIDOV (**21**) there are only three papers that show a systematic analysis of the method and of the application of the nucleal stain and the difficulties encountered in its use. FEULGEN (**8**) outlined the general method and pointed out the extent and general limitations of its application. MILOVIDOV (**20**) analyzed the causes of a negative nucleal reaction and gave reasons for it, all of which are sound except for his conclusions regarding tannins. BAUER (**1**) investigated the effects of various fixatives and gave tables and graphs showing the time of hydrolysis that would give the best results with the various fixatives. Yet the recent literature is full of contradictions as to the effect of fixatives and time of hydrolysis on the reaction.

To try to clear up some of the confusions and contradictions regarding the nucleal stain, and to demonstrate its value in cytology, the following points were investigated.

a) The effect on the Feulgen reaction of various fixatives and of the different ingredients in these fixatives, thus extending the investigations of BAUER.

b) The effect of such substances as tannins, which have been suggested as interfering with the reaction.

c) The non-nucleal stains produced by leuco basic fuchsin, which may accompany and confuse the results of the nucleal stain.

d) The use of the nucleal stain in making clear such chromosome structures as the satellites and nuclear phases such as zygotene, diplotene, and metaphase stages, which cannot be studied in many species by ordinary methods of staining.

e) The use of the nucleal stain for the study of chromosome struc-

ture, and the conclusions that may be drawn as to the relation between structure and chemical composition.

f) The possibilities of the use of the nucleal stain to study the finer details of the structure of the chromonemata following proteolytic digestion; that is, the tryptic digestion method of CASPERSSON (**6**).

g) The development of new techniques made possible by the Feulgen nucleal reaction.

The first three of these questions are dealt with in the present paper.

Reagents

Reagents of the proper type are one of the prerequisites to success with the nucleal reaction, thus their preparation is important. The length of the period of hydrolysis is perhaps the most critical point of the reaction. The optimum of hydrolysis varies with the concentration of the acid and the temperature at which hydrolysis is carried out (**9**). The acid must therefore be of a standardized concentration and the temperature must be constant, since the procedures are based on these factors. Normal hydrochloric acid is used, and 60° C. has been adopted as a standard hydrolyzing temperature because it allows the optimum to be reached with a fairly short period of hydrolysis and it is not high enough to damage the structure of the nucleus.

The necessity of having a completely decolorized fuchsin sulphurous acid or leuco basic fuchsin has been mentioned. COLEMAN (**7**) gives the method of preparing such a solution. The SO_2 water, since it is not an integral part of the reaction but merely a bleaching rinse, need not be so carefully prepared as the other reagents. FEULGEN (**8**) gives the method of preparing this solution, but the use of potassium metabisulphite as suggested by DE TOMASI (**28**) produces a more satisfactory solution.

Experimentation

Our present knowledge of the effects of fixing reagents on the Feulgen stain we owe almost exclusively to BAUER (**1**), who tested some twenty-four fixation mixtures in this connection. BAUER used chiefly animal cells (spermatocytes of grasshoppers), and showed

the effect of hydrolysis on the intensity of the stain with the various fixatives. While BAUER compared the results obtained by the nucleal stain with those obtained with a standard stain such as iron haematoxylin, he confined his tests in plants to sections of onion root tips, and of course did not attempt the use of the stain in the elucidation of chromosome structure. His attempts to account for the marked differential action of the various fixatives as affecting the time of hydrolysis do not seem very successful, while the method used (observation under the microscope) does not allow for a ready and rapid comparison of these effects. As he freely admits, the subjective element entered to no small degree into the construction of his graphs. It was felt therefore that BAUER'S work should be reviewed and extended in an attempt to obtain a more objective picture of events, and at the same time to elucidate more fully the cause for the differential action of fixing reagents.

I. IN VITRO EXPERIMENTS

The in vitro experiments of FEULGEN were conducted in solutions of thymus nucleic acid in test tubes. Obviously such a method is out of the question where a fixing reagent is to be added, for it is impossible to separate it from the thymus nucleic acid solution after the fixing action has taken place. It was necessary therefore to include the thymus nucleic acid in a gel medium such as agar, which on cooling could be treated in a manner comparable with that used for plant tissue.

The agar blocks used in the in vitro experiments were made by adding 0.1 gm. of sodium nucleinate in 10 cc. of water to 100 cc. of boiling 5 per cent Bacto purified agar. This mixture was stirred for a few minutes, poured into plates, and after cooling and solidifying, cut into blocks. The blocks were actually cubes, with sides about 5 mm. in length, and the size was kept as uniform as possible so that accurate comparisons could be made. The agar blocks provide a convenient carrier for the nucleic acid as they can be passed through the various solutions without dissolution or leaching out of the acid. Also they permit macroscopical observation, as the vials containing the agar blocks can be placed side by side and whole series showing narrow differences in color can be compared at a

glance. In all experiments, the results of the agar blocks when compared with those obtained in cytological preparations (pollen mother cell smears of *Tradescantia virginiana*) were found to be identical.

The fixatives used represent four different types and belong to the four main groups of fixatives:

1. Feulgen's fixative (sublimate-acetic)
2. Carnoy's fluid (alcohol-acetic)
3. La Cour's 2 BE (chrom-osmic-acetic)
4. Belling's fixative (chrom-formol-acetic), a modification of the San Felice and Navashin type

RESULTS.—The first experiment with the agar blocks was to determine what effect the time of fixation had on the stain. Three series were run with each of the fixatives, the periods being 1, 24, and 48 hours, followed by 18 hours' washing with running water. In each series the periods of hydrolysis were 0, 1, 5, 10, 15, 20, 25, and 30 minutes at 60° C. with N HCl. The sample with no hydrolysis was used as a check to see whether any color was caused by some constituent of the fixative such as formalin. The agar blocks were left in leuco basic fuchsin for 30 minutes and allowed to bleach 24 hours in SO_2 water, during which period the solution was changed three times. In all three series no differences in the staining after a particular fixative could be observed. Thus the duration of fixation has no effect on the intensity of the stain. In the light of this, the results presented in this section are based on 24 hours' fixation and the preceding staining schedule.

The second experiment was to determine the optimum time of hydrolysis with the four fixatives. Along with this, a series of nucleic acid blocks with which no fixing agent had been used were treated in the same way, to serve as a check. The results of these experiments are shown in the graphs of figure 1. Agar blocks free from nucleic acid were also treated in the same manner to make sure that the colors produced were due to the added acid and not to any nucleic acid that might be in the agar itself.

The first type of curve (fig. 1*A*) is shown by the series with no fixation and by the Feulgen and Carnoy fixative series. These two fixatives bring about no change in the nucleic acid as far as can be

measured by the Feulgen reaction. The curve shows a rapid rise in stain intensity with increasing hydrolysis, until at 4 minutes a strong stain is reached. This maximum is maintained through the range from 4 to 8 minutes, then there is a slight dropping off in color to medium strong at 9 minutes and to medium at 10 minutes. The dropping off continues gradually until 15 minutes, then less rapidly to 20 minutes, and at 30 minutes' hydrolysis no stain is perceptible.

The second type of curve (fig. 1*B*) is shown by the La Cour 2 BE and Belling fixative series. This curve is very different from the other type. Instead of the color beginning to shade off at 9 minutes of hydrolysis it continues strong and heavy right through and past 30 minutes. Another difference is that the maximum is not reached so soon. At 5 minutes' hydrolysis the stain is medium strong; at 6 minutes it is strong, and this maximum strength is maintained until about 50 minutes when a falling off in intensity occurs.

This difference is apparently caused by some constituent or constituents present in the La Cour 2 BE and Belling fixatives but not present in the Feulgen and Carnoy fixatives. The only constituent common to both of the former fixatives but not present in the latter two is chromic acid. To make sure that chromic acid is the agent responsible, a series in which 1 per cent chromic acid was used as the fixative was treated in the same way. This produced the same type of staining curve as the La Cour 2 BE and Belling fixatives. Each of the other ingredients of the fixatives was tested individually and all produced the same type of curve as the normal or Feulgen and Carnoy types. There can therefore be no doubt that chromic acid and it alone is responsible for the marked difference in the staining curves.

DISCUSSION.—The in vitro experiments have given results confirmatory in a general way to those obtained by BAUER. There are differences, probably due mainly to the fact that it is easy to observe slight color differences in the agar blocks while in microscopical preparations their exact determination is difficult. In addition, the in vitro experiments permit an analysis of the action of the various ingredients of the fixatives. This BAUER did not do, and he failed to recognize that chromic acid is the cause of the two types of

staining curves. Thus his tentative explanation for the fixation effect cannot stand. To facilitate comparison and discussion, BAUER'S curves are reproduced here as figure 2.

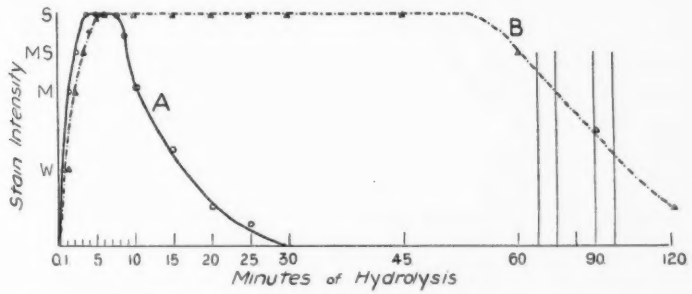

FIG. 1.—Staining curves as shown by nucleic acid impregnated agar blocks: *A*, Feulgen fixative (sublimate-acetic) and Carnoy (alcohol-acetic); *B*, La Cour 2 BE (chrom-osmic-acetic) and Belling (chrom-formol-acetic).

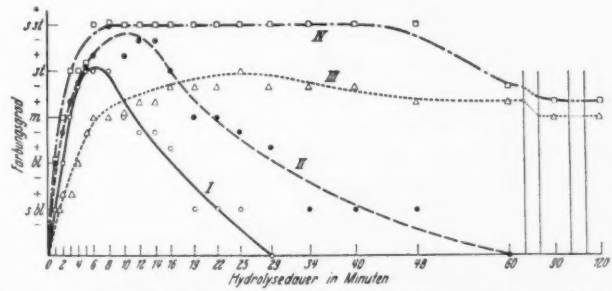

Abb. 1. Zusammenhang zwischen Hydrolysedauer und Färbungsgrad. Kurve I ○—○ Sublimateisessigfixierung; Kurve II ●·····● HELLY-Fixierung; Kurve III △·····△ FLEMMING-HEITZ-Fixierung; Kurve IV □·····□ SANFELICE-Fixierung.

FIG. 2.—Reproduction of BAUER'S curves. Zeitschr. Zellf. Mikr. Anat. 15:231. 1932

In BAUER'S curves, curve I corresponds to the Feulgen fixative curve and also to the Carnoy curve. Figure 1*A* represents the curves for both these fixatives. Curve II cannot be considered because the Helly fixative was not used in my experiments. Curve III corresponds to the curve for the La Cour 2 BE fixative and curve IV to the Belling fixative. These are both represented by figure 1*B*, since

with the agar blocks these two fixatives produce the same type of curve. The main difference in the two sets is that with the agar block experiments the maxima are all the same, whereas they vary in BAUER'S experiments. This can probably be explained on the basis of the difficulty of distinguishing fine color differences in microscopical preparations. Why, in curve III, BAUER ignored the course of five points to go up to a maximum at 20 minutes is not understood. This curve, if it followed the majority of points, would show a flat topped maximum as in curve IV, just as is shown by the curves for a similar fixative in the agar block experiment.

BAUER'S explanation that the rising phase of the curve is due to the gradual splitting off of the purine bodies by hydrolysis is chemically correct. For the falling off of the curve he gives two possibilities. First, it may be due to a further degradation of the thymin acid molecule (the part of the thymonucleic acid molecule left after the splitting off of the purine bodies); second, it may be due to the destruction of the union between nucleic acid and the protein components of the nucleoprotein. The second explanation is not tenable because the falling off of the stain occurs with the agar blocks just the same as it does with nuclei, and the nucleic acid is not bound to nucleoproteins in the agar blocks.

In the first explanation BAUER does not make clear what he means by a further degradation of the thymin acid molecule. It cannot be due to a chemical degradation of the aldehyde or color-producing groups, because a solution of thymus nucleic acid in a test tube is still capable of reaction and producing color after 8 hours of hydrolysis. To test this question further a series of nucleic acid blocks were hydrolyzed for 5–30 minutes in vials. The HCl from each vial was poured off and tested with leuco basic fuchsin at the same time that the corresponding agar blocks were tested. The acid in the vials showed a stain intensity which was the reciprocal of that of the agar block series. In other words, the aldehyde had passed out of the blocks into the hydrolyzing liquid. This experiment, together with the fact that 8 hours' hydrolysis does not bring about chemical degradation of the aldehyde groups, shows that a linkage is broken somewhere. Since the breakdown of the bonds is not between the nucleic acid molecules and nucleoproteins, and not within the alde-

hyde groups, it must be a breakage of the union between the aldehyde groups and the remainder of the thymonucleic acid molecule. Perhaps future biochemical investigation will reveal the details of this.

With the second type of curve the agar block experiments have shown that retention of the stain after long hydrolysis is due to chromic acid. Thus BAUER'S explanation, which is based on the supposed unavailability and resulting unreactability of nucleic acid due to dense precipitation of the nuclear proteins brought about by the homogeneous fixation (gelatinization) of osmic and formaldehyde fixatives, as contrasted with the coarse coagulation of other fixatives, falls to the ground. BAUER, however, does not overlook the possibility of the effect of chromic compounds and the action of a specific chemical factor. The before-mentioned experiments to determine the reason for the fall in the curves would suggest that chromic acid acts in some way to strengthen the binding between the various groups of the nucleic acid molecule, allowing them to resist for a longer time the disintegrating effect of hydrolysis. This remains a subject for future investigation.

Besides the effect of chromic acid, the agar block experiments show, as BAUER has pointed out, that the Feulgen nucleal stain is not limited to material fixed in sublimate fixatives, which are in general unsatisfactory for cytological purposes. FEULGEN'S precautions regarding the use of fixatives containing formalin or substances that are strong oxidizing agents like chromic acid are not necessary, provided these substances are in the concentration normally used in fixatives. The effects of the various other constituents of fixatives will now be discussed.

Osmic acid does not interfere with the stain if the darkening due to it is completely bleached out with hydrogen peroxide before hydrolysis. When it is not completely bleached out the stain is less intense.

Formalin, being itself an aldehyde and capable of reaction with leuco basic fuchsin to give the same color as the nucleal stain, was considered by FEULGEN to be an unsatisfactory constituent of fixatives as the color produced by it on the surface of tissues and in the cytoplasm would be apt to obscure the stain of the nucleus.

Such objectionable effects can be avoided by thorough washing following fixation with a fixative containing formalin. Proof that formalin can be thoroughly washed out is provided by the agar block experiment with 20 per cent formalin, in which the normal staining series is produced. Thus the reports of BERG (**3**), FEULGEN (**8**), HUBER (**14**), and VOSS (**29**) that formalin is an unsatisfactory constituent of fixatives seem untenable.

Some workers have claimed that fixatives with a low acetic acid content are best for use with the Feulgen reaction. SEMMENS and BHADURI (**24**), for their work on the differential staining of nucleoli and chromosomes, suggest Navashin's or Levitsky's fixatives with the acetic acid cut down to a minimum. Why, they do not say. WHITAKER (**32**), working with various plant materials, states that in his experience it is quite important that a fixative be employed which contains a minimum of acetic acid, and suggests Benda's (low acetic) fluid. In this laboratory we can see no difference in the intensity of the stain after using fixatives containing either a low or a high content of acetic acid. In fact some of our clearest and sharpest preparations were fixed in Carnoy's fluid (3:1), which contains 25 per cent of acetic acid. In order to test the effect of various concentrations of acetic acid on the nucleal stain, nucleic acid impregnated agar blocks were nucleal stained, after 24 hours' treatment, in 1, 5, 10, 25, 50, 75 per cent, and glacial acetic acid. All strengths up to and including the 50 per cent acetic acid gave the full maximum stain. With 75 per cent a slight reduction in the intensity of the stain was perceptible, and with glacial acetic acid a medium strong stain was produced. Thus all the evidence indicates that acetic acid has no deleterious effect on the Feulgen nucleal stain. As has been pointed out, the same statement applies to the other constituents of the commonly used cytological fixatives.

II. EXPERIMENTS WITH PLANTS

In order to determine whether the nucleal stain is satisfactory for all types of plants and whether fixatives have the same effect as with the agar blocks, a similar series of experiments was carried out on various members of the different divisions of the plant kingdom. Particular attention was paid to those plants in which a nega-

tive reaction had been reported. Nuclei from as many different types of cells as could be obtained were studied in order to compare the stain in nuclei of different organs of the plant.

RESULTS.—The following plants were fixed in the four fixatives and hydrolyzed and stained in the same way as the agar blocks.

Cyanophyceae
Oscillatoria—stained granules
Diatomeae
Navicula, etc.—somatic nuclei
Chlorophyceae
Ulothrix zonata—somatic, zoospore, and gamete nuclei
Cladophora—somatic nuclei
Spirogyra, 4 species—nuclei
Desmids, *Closterium*, etc.—somatic nuclei
Nitella batrachosperma—somatic, egg, and sperm nuclei
Phaeophyceae
Fucus spiralis—somatic, egg, and sperm nuclei
Phycomycetes
Mucor—hyphal, immature, and mature spore nuclei
Ascomycetes
Geopyxis cupularis—hyphal, immature, and mature spore nuclei
Basidiomycetes
Aleurodiscus canadensis (Jackson, in ed.)—dividing and resting nuclei
Bryophyta
Riccia natans—spore mother cell and spore nuclei
Marchantia polymorpha—somatic, sperm, and egg nuclei
Mnium—somatic, sperm, and egg nuclei
Pteridophyta
Pteris cretica crustata—spermatozoid, prothallial, and somatic nuclei
Adiantum cuneatum—spermatozoid, prothallial, and somatic nuclei
Gymnosperms
Pinus austriaca—somatic, egg, and pollen mother cell nuclei
P. sylvestris—somatic, egg, and pollen mother cell nuclei
Angiosperms
Lycopersicum esculentum—somatic and pollen mother cell nuclei
Typha latifolia—somatic nuclei
Tradescantia virginiana—somatic and pollen mother cell nuclei
T. paludosa—somatic and pollen mother cell nuclei
T. reflexa—somatic and pollen mother cell nuclei
Lilium harrisii—somatic, pollen mother cell, and egg nuclei
L. longiflorum var. *formosum*—somatic, pollen mother cell, and egg nuclei
Also many other species of angiosperms.

DISCUSSION.—A positive nucleal reaction was obtained in all the plants listed. In the majority of cases the type of staining curve with a particular fixative corresponds with that for the similarly treated agar blocks. The only plant that did not conform more or less to the typical staining curves was *Spirogyra* spp.

In the case of *Spirogyra* the maximum is shifted to a longer period of hydrolysis (15–20 minutes). YAMAHA and SUEMATSU (**33**) give for this form a hydrolysis period of 5–10 minutes, and GEITLER (**12**) 5–8 minutes; in my experiments this range of hydrolysis did not produce a good stain. A negative reaction for *Spirogyra* has been reported by HUREL-PY (**15**), KIESEL and DOINIKOWA (**16**), MILOVIDOV (**20**), PETTER (**22**), and SHINKE and SHIGENAGA (**26**). GEITLER was the first to detect a positive stain. He investigated the structure of the resting nucleus and found that the stain was confined to widely separated small dotlike and rod-shaped bodies, the pro-chromosomes or chromocenters. It is possible that the negative results previously reported for *Spirogyra* are due to the fact that the investigators overlooked the small chromocenters, or perhaps they did not hydrolyze long enough to make them evident.

Marchantia was the only plant in which difficulty was experienced in obtaining a satisfactory stain with all types of nuclei. The staining curves were of the normal type but the maximum stain was weak. This weak stain occurred in all types of nuclei, even those of spermatozoids, which usually take the heaviest stain owing to the compactness of the chromatin. Results such as this would lead one to suspect a chemical difference in the nuclei of this plant.

It was observed in plants with which it was difficult to obtain an intense stain owing to the dispersed nature of the staining material that the best results were obtained after the La Cour and Belling fixatives. These fixatives have also the advantage that the period of hydrolysis is not limited to a narrow range as with the others. BAUER noted this and brought out another interesting point in favor of these fixatives. The Feulgen nucleal stain has frequently been criticized on the basis that the acid hydrolysis will perhaps destroy some of the finer structural details of the nucleus. BAUER points out that material fixed with fixatives which prolong the staining will maintain the true structure much longer because they resist

the effect of hydrolysis for a greater length of time. This applies to the chromic acid fixatives. In no instance in my work on the structure of chromosomes has any destruction of the finer details of structure been noted. Of course only short hydrolysis periods were used, and no doubt structure would be destroyed with longer hydrolysis, especially with the Carnoy fixative. The various experiments have shown that a nucleal stain is possible with all kinds of plants, and the intensity of this stain and thereby its usefulness is dependent on the interrelation of two factors, fixation and hydrolysis.

III. Period of hydrolysis

The time of hydrolysis is the cardinal factor in the Feulgen technique. Whitaker (**32**) remarks that the time allowed for hydrolysis is one of the critical points of the technique, but then makes the statement: "In general, it is governed by the length of the period of fixation, i.e., if fixation lasts 30 minutes, hydrolysis should extend over the same period." Thirty minutes' hydrolysis will produce a good stain with the chromic acid fixatives such as Benda, but not with those lacking chromic acid, and if fixation extends over hours, hydrolysis could not be carried on for the same length of time. Feulgen (**8**) gives 4 minutes as the optimum time of hydrolysis and this is correct for the sublimate-acetic fixative he was using. Bauer (**1**) gives the optimum time for twenty-four fixatives. The agar block experiments have demonstrated that the differences are due to chromic acid. From these experiments two generalizations can be drawn: (1) with fixatives containing chromic acid the optimum hydrolysis period for maximum stain is 5–30 minutes; (2) with fixatives lacking the acid it is 4–8 minutes. Exceptions to this general rule are known, especially in lower plants. For example, in the case of *Spirogyra*, with fixatives lacking chromic acid the optimum hydrolysis period is shifted up to 15–20 minutes. Thus in cases where no stain is produced in the normal hydrolysis range, a stain can often be obtained by increasing the time of hydrolysis. Also with large quantities of material which lower the temperature of the acid, and with large masses of tissue where it takes some time for the acid to penetrate to the interior and raise the temperature there, it is necessary to increase the time of hydrolysis. From the results

of the various experiments it can be concluded that with higher plants and with most of the lower ones a satisfactory nucleal stain can be brought about by 5 minutes' hydrolysis, no matter what fixative is used.

IV. Possibilities of a negative nucleal reaction

A negative nucleal reaction could result from the action of compounds which can be designated as stain inhibitors. By stain inhibitors are meant those compounds which have been reported as hindering or preventing the occurrence of a Feulgen reaction, thus

TABLE 1

Percentage chromic acid concentration	Fixation		
	10 minutes	1 hour	24 hours
1	S*	S	S
2	S	S	S
5	S	S	Smear gone
10	S	S	" "
25	Smear gone	Smear gone	" "
50	" "	" "	" "

* S=strong stain.

resulting in a partial stain or none at all. The first of these compounds to be considered is chromic acid. Yuasa (**34**), working with ferns, claims that above a certain limiting percentage of chromic acid (1 per cent for most species) a negative reaction results. Attempts to duplicate Yuasa's work, using *Pteris* and *Adiantum* leaves, yielded results that do not agree with his. At concentrations of 25 per cent and above the leaves disintegrated, as did smears of *Tradescantia virginiana* pollen mother cells. Table 1 shows the results with smears, all with 5 minutes' hydrolysis.

The nucleic acid impregnated agar blocks present a clearer picture of what happens (table 2).

Obviously the partially or completely inhibiting action of chromic acid at high concentrations is due to its strong oxidizing power, which destroys the nuclear material. Yet to do this it requires a very high concentration acting for a considerable time. At concen-

trations such as are used in fixing reagents, chromic acid has no deleterious effect; on the contrary it postpones the falling off of the stain with increased hydrolysis.

Tannins are considered in many cases to be the cause of a negative nucleal reaction with plants. MILOVIDOV (**20**) pointed out that tannins are present in many plant cells, particularly epidermal cells, and that in these cells there is a weakening of the stain. On this basis he would explain the results of BOAS and BIECHELE (**4**), who reported that the nuclei of the epidermal cells of 138 out of the 224

TABLE 2

PERCENTAGE CHROMIC ACID CONCENTRATION	FIXATION		
	10 MINUTES	1 HOUR	24 HOURS
1	S*	S	S
2	S	S	S
5	S	S	No stain
10	S	S	" "
25	S	M	" "
50	S	W	" "

* S=strong stain; M=medium stain; W=weak stain.

plants investigated showed a negative nucleal reaction. As TISCHLER (**27**) points out, perhaps these negative results could better be explained on the basis of faulty technique, failure to recognize small amounts of nucleic acid, slight chemical differences, and unsuitability of the materials and methods. MILOVIDOV seems convinced that tannins interfere with the nucleal reaction by hindering the penetration of reagents. He substantiates his claims by considerable experimentation. In cases where he can trace the presence of tannin to an individual cell he finds a weakening of the nucleal reaction. With the series of angiosperms which he investigated, two showed a negative nucleal reaction and others a weak reaction. These occurred in plants known to contain considerable quantities of tannins. In *Fucus vescoides* he reports that there is a weakened nucleal reaction in cells surrounded by tannins, but this could not be confirmed in my own work with *Fucus*. To test further the hindering

action of tannins, MILOVIDOV soaked slides with sections on them in 2 per cent tannic acid and found a weakening of the stain. Likewise he showed that preparations treated in this way failed to take up gentian violet and methyl green. In presenting this impressive series of experiments and facts to support his claims regarding the inhibiting action of tannins, MILOVIDOV concludes by stating that more experimental work is necessary to confirm his assumptions.

In order to investigate thoroughly the question of the effect of tannins on the nucleal reaction, extensive experiments with plant material and agar blocks were carried out. In all cases the material was soaked in 2 per cent tannic acid for 24 hours, rapidly rinsed in water to remove only the excess of tannic acid, then transferred directly to the HCl for hydrolysis. Other concentrations of tannic acid were tried but the effect was the same, so a 2 per cent solution was used as standard. The results of these experiments are shown in the graphs of figure 3. Each curve shows the results for nucleic acid impregnated agar blocks as well as pollen mother cell smears of *Tradescantia paludosa*, since both produced the same results.

Graph 1 represents the curve produced by the Feulgen and Carnoy fixatives. The tannic acid curve is very different from the normal type: the maximum is not reached until 8 minutes of hydrolysis, and instead of falling away there it continues through to 30 minutes of hydrolysis, where a slight dropping off begins. This is similar to the effect produced by chromic acid. Tannins certainly do not inhibit the stain, except perhaps slightly at first.

Graph 2 shows the curves with La Cour 2 BE fixative. Here the only difference in the two curves is that the maximum is not reached until 12 minutes of hydrolysis. Again there is a slight inhibiting effect at first.

Graph 3 is for the Belling fixative and here there is a definite weakening of the stain all through the range of hydrolysis. The maximum stain is never attained; a medium stain is reached at 10 minutes and this level is maintained up to 30 minutes and beyond.

Comparing these three graphs and the constituents of the four fixatives that produce them, formalin is the only constituent that is present in the Belling fixative and lacking in the others. Graph 4 shows the type of staining with 20 per cent formalin. The inhibition

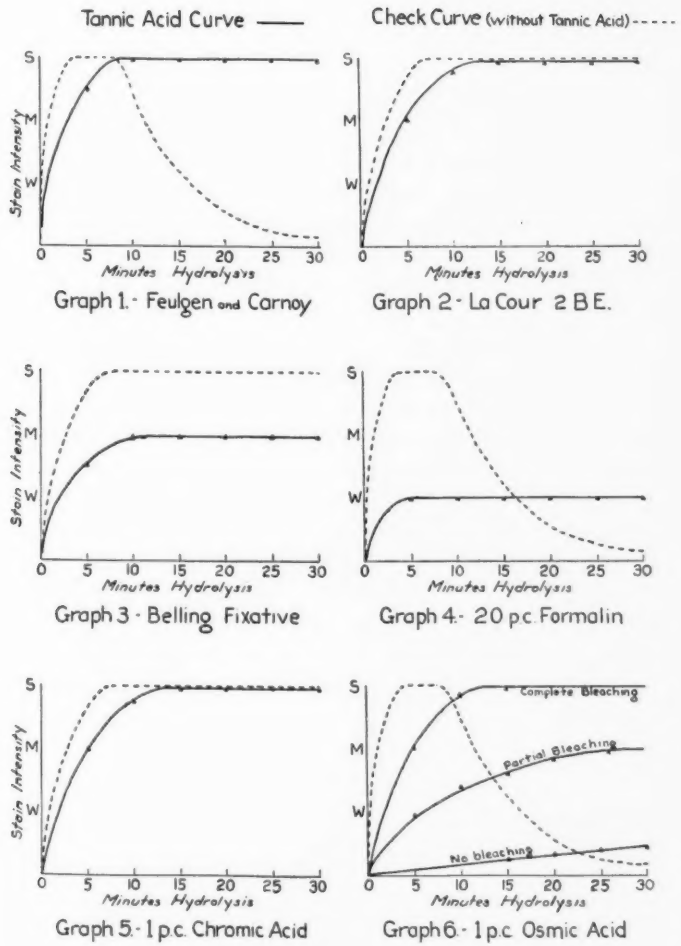

FIG. 3.—Effect of tannic acid on staining curves as shown by nucleic acid impregnated agar blocks and plant materials with various fixatives. Dotted line, check or normal result; solid line, tannic acid curve.

of the stain here is even greater than with the Belling fixative, as only a weak color is produced. This difference is probably due to the fact that 20 per cent formalin represents a higher concentration than that in the Belling fixative. That this general weakening is due to formalin alone can be seen by examining the various graphs. Graph 5 shows the type of curve produced by 1 per cent chromic acid and this is the regular chromic acid curve. The other ingredients of the fixatives—1 per cent potassium bichromate, 6 per cent sublimate, 5 per cent acetic acid, and 50 per cent alcohol—show the same type of curve as that for the Feulgen and Carnoy fixatives (graph 1).

Graph 6 is for 2 per cent osmic acid. The three tannic acid curves show the different amounts of inhibition produced by different amounts of bleaching of the osmic acid with hydrogen peroxide. The more complete the bleaching the sooner the maximum is reached. Lack of complete bleaching of the osmic acid probably explains the delay in reaching a maximum stain with La Cour 2 BE.

These results are in partial agreement with those of MILOVIDOV. In all cases there is a slight inhibition of the stain at the low periods of hydrolysis. These are the hydrolysis periods (4 to 8 minutes) normally used in cytological work, but the weakening is so slight that it may be ignored. The important point is that with fixatives containing formalin the weakening of the stain in its presence is serious. This is the sort of effect that MILOVIDOV reports, but he was not using fixatives which contain formalin. Thus the inhibiting action of tannin on the nucleal stain is not so serious as MILOVIDOV considers it to be.

Strong alkalies will inhibit the nucleal stain by virtue of the fact that they destroy the nuclear material. This was observed with root tips treated after hydrolysis with hot alkali to remove the pectic acid of the middle lamella in order to bring about a separation of the cells for the squash technique. This is discussed by HILLARY (13), together with the fading of the stain which results when corn syrup diluted with acetic acid is used as a mounting medium. This is the only instance as far as is known of the Feulgen nucleal stain fading.

The possibility of a negative nucleal reaction being reported owing to failure of the observer to see the widely separated chromocenters of resting nuclei has been discussed in the case of *Spirogyra*. This probably explains the report by WALTHER (30) of a negative nucleal reaction with the egg nuclei and a positive reaction with the sperm nuclei of *Nitella*. In my investigations the egg nucleus of *Nitella* showed small widely separated chromocenters. Another case of this sort was found in the nuclei of the germinating radicle of *Typha latifolia*, where the chromocenters were so small that the stain was hardly discernible; thus it would be easy to overlook them. The same was found to be the case with fungi; in most cases there was a large nucleus with small chromocenters distributed around the nucleolus and the periphery of the nucleus. A weak stain due to a great dispersion of the staining material in a large nucleus is strikingly demonstrated in *Fucus*. Here the ratio of volume of sperm to egg nucleus is 1 to 700, with presumably the same amount of staining material in each. The sperm nucleus shows a heavy stain while the egg nucleus shows a weak one because the stain is confined to the chromocenters, which are distributed over the large volume of the nucleus. A similar observation was made with the egg nucleus of *Pinus*, except that this nucleus shows a reticulate type of structure. A review of the literature dealing with this question in both plants and animals has been given by MILOVIDOV (20).

The final possible cause of a negative nucleal reaction is a chemical alteration in the thymonucleic acid. MILOVIDOV (20) has reviewed the literature dealing with this problem. In animals there is strong evidence to support the view that there is a chemical alteration or synthesis of nucleic acid during development. With plants the evidence is not so complete, but there are indications of it. WESTBROOK (31) finds a very weak reaction with the sporangia of *Rhodymenia palmata* during the growth stage. SHIMAMURA (25) reports a negative reaction with the reticulum of sperm and egg nuclei as well as with the central, ventral canal cell, and conjugating nuclei of *Cycas* and *Ginkgo*. This could perhaps be explained by the wide dispersion of the chromatin in the egg nuclei but not with the others, especially the sperm nuclei. BERG (3) claims a chemical difference to explain the negative reaction of the megagametophyte

nuclei in *Lilium*, but careful investigation of the megagametophyte nuclei at all stages of development has shown only a strong positive reaction, even in a well dispersed reticulum. A dispersion of the chromatin will not explain the very weak stain in all types of nuclei in *Marchantia*. The only explanation for this, excluding the possibility of stain inhibiting substances, is a chemical difference, and such could easily be the case considering the complexity of the nucleic acid molecule.

The possibility of the nucleal stain being obscured by colorations produced by leuco basic fuchsin in structures other than the nucleus was investigated. These colorations are produced (with or without hydrolysis) in lignin, suberin, cutin, starch, glycogen and other polysaccharides, owing to the aldehyde groups present in these compounds. (With no hydrolysis, this reaction is merely SCHIFF'S test for an aldehyde.) The details of such stains have been discussed by BAUER (**2**), MARGOLENA (**18**), MIDDENDORF (**19**), and MILOVIDOV (**20**), so they will not be treated fully here. In only one case, *Vaucheria*, was the color in the cell wall strong enough to interfere with observation of the nucleal stain. In a few forms some trouble was caused by the plasmal reaction, which is a coloration of the cytoplasm. It is due to the action of leuco basic fuchsin on lipoids, which are retained by sublimate fixation (**8**) and chromic fixation (**5**). This can be overcome if the material is treated 24 hours with 95 per cent alcohol. These non-nucleal stains rarely interfere with the nucleal stain and need not be considered as drawbacks to the use of the nucleal reaction. On the other hand they provide additional uses for the stain; for example, the color produced by it on lignin is superior to that with phloroglucin.

The previous experiments and discussions show that there are various explanations possible for a negative nucleal reaction. These can be grouped under five headings, following MILOVIDOV'S classification:

1. Unsuitable general methods; that is, insufficient fixation and preparation, or unsuitable object (presence of thick cell walls or heavy cuticle or fats which would hinder observation of the nucleus).

2. Incompleteness in the methods employed in the nucleal reac-

tion; that is, wrong period of hydrolysis, temperature of hydrolysis, and concentration of HCl.

3. Presence of materials which hinder the normal process of the reaction; that is, neutralization or binding of HCl; binding of the liberated aldehyde groups; disturbances of the penetration of reagents by resins, tannins, and slime substances (a slight effect).

4. Strong dispersion of a small amount of chromatin in a large nuclear volume.

5. Chemical alteration of thymonucleic acid.

The first four have been shown to be the reasons for negative results in many cases; the fifth, chemical alteration, while a reasonable possibility, still remains to be thoroughly investigated.

Summary

1. In vitro experiments with nucleic acid impregnated agar blocks, using four different types of fixatives, show that the Feulgen nuclear stain gives two types of staining curves, depending on the presence or absence of chromic acid in the fixative.

2. With fixatives not containing chromic acid the maximum stain is produced by hydrolysis at 60° C. extending from 4 to 8 minutes; after that time a gradual falling off of the stain takes place, until at 30 minutes no further stain is visible. With fixatives containing chromic acid the maximum stain is produced by hydrolysis at 60° C. for 5–30 minutes.

3. That retention of the stain with increased hydrolysis is due to chromic acid has been demonstrated by testing the individual ingredients of the fixatives separately. In this case only chromic fixation produces the type of staining curve with the prolonged maximum.

4. None of the ingredients of the fixatives, if present at the concentration normally used, interfere with the stain. Chromic acid at high concentrations (above 5 per cent) and acting for a considerable length of time will prevent the stain, owing to destruction of the nuclear material.

5. Tannins, which have been considered by MILOVIDOV to inhibit the stain, have the same effect as chromic acid in retaining it, except

when a fixative is used that contains formalin. In that case there is considerable weakening of the stain.

6. Various plants from the different divisions of the plant kingdom, when treated with the four fixatives in the same way as the agar blocks, give the same results. A stain, usually a strong one, is obtained in all cases. In some cases the stain is not pronounced owing to dispersion of the chromatin in widely separated chromocenters or to the presence of a diffuse reticulum in a large nucleus. In one case, *Marchantia*, all types of nuclei show only a weak stain, suggesting a chemical difference, at least as regards part of the nucleic acid present. These are considered to be possible explanations of the frequently reported negative results.

7. It can be concluded in general that for the production of a satisfactory stain 5 minutes' hydrolysis with any of the regularly used cytological fixatives is sufficient.

The writer wishes to express his gratitude to Dr. L. C. COLEMAN, who suggested this problem and rendered generous assistance throughout its progress.

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ONTOGENETIC AND ANATOMICAL STUDIES OF THE FLOWER AND FRUIT OF THE FAGACEAE AND JUGLANDACEAE[1]

LADEMA MARY LANGDON

(WITH SEVENTY FIGURES)

Introduction

A critical study has been made of certain of the woody ament-bearing angiosperms, with particular attention to the Casuarinaceae, Fagaceae, and Juglandaceae. Life histories have been followed in detail, and all conclusions relating to floral structure have been fully supported by comparative studies of vascular organization. The principal object has been to determine what structures are primitive and what are reduced; that is, to secure additional data bearing upon the perplexing problem of the relationship of the various members of these families to one another and to the rest of the angiosperms.

An inconspicuous bractlike perianth, catkin development, and the frequency of chalazogamy placed the Casuarinales, Juglandales, and Fagales among the most primitive in ENGLER'S Archichlamydeae, preceding such petaliferous groups as the Rosales and Magnoliales. This position has been challenged by certain morphologists and systematists (**3, 4, 9, 10, 12, 24, 35**), who interpret the bractlike perianth and catkin development, both adaptations for wind pollination, as specialized rather than primitive features. The frequent occurrence of a syncarpous gynoecium among many of the Amentaceae is also considered by them to be at variance with the primitive position accorded these groups in the ENGLER system. Developed on the hypothesis that plants with a perianth associated with other primitive characters (floral and anatomical) are more ancient than plants lacking a perianth, HUTCHINSON'S new system

[1] A section of this report was presented before the General Section of the Botanical Society of America at Indianapolis; December, 1937. The investigation has been aided by grants from the Committee on Grants-in-Aid, National Research Council, and the Faculty Research Fund, Goucher College.

(24) derives the Amentiferae from the Magnoliales: the Juglandales along the line of the Celastrales and the Sapindales; the rest of the Amentaceae as reductions from the Rosales through the Hamamelidales; with the Casuarinaceae as illustrative of extreme reduction, possibly in adaptation to dry climatic conditions. In recent phyletic studies, BROWN (11) likewise inclines to the derivation of the Juglandales from the Rosalian line by way of the Sapindales, and interprets the cup-shaped torus characterizing the Rosales and many of their descendants as a nectary located between the carpels and the stamens. In the case of the Juglandales, he assumes a degeneration of the nectary along with the development of adaptations for wind pollination, a tendency to be observed also in the Aceraceae.

On the other hand, RENDLE'S taxonomic studies (34) support the theory—as the one most in accord with present knowledge—that the Casuarinales, Fagales, and Juglandales are surviving representatives of one or more diverging lines of development from some early angiospermous type or types now extinct, very likely with bisexual hypogynous flowers and bractlike perianths. Summarized, his conclusions are that the complete absence of the herbaceous habit, the frequency of the chalazogamic method of fertilization, the marked interval between pollination and fertilization, as well as the long geological history of these orders suggest that in these forms we are concerned with isolated remnants of relatively ancient groups which have left no descendants among the more highly developed orders of our present flora. A similar conclusion appears to have been reached by NAVASHIN and FINN (30) with respect to the Juglandaceae.

Recent researches in inflorescence anatomy and morphology in families of the Amentiferae, including the Salicaceae (20), Betulaceae (1, 2), and Juglandaceae (28), have emphasized specialization and reduction in these groups; and those who have gone into the details of floral structure have been in general agreement in characterizing the inferior ovary as "appendicular." Opposed to this interpretation of the epigynous condition in flowering plants in general are the developmental studies of THOMPSON (40, 41, 42), those in paleobotany by THOMAS (38, 39), as well as the floral studies of others (13, 22, 23, 37) which give substantial support to the "re-

ceptacular" origin of the inferior ovary. Critical discussions of the theories of THOMAS and THOMPSON have appeared (**5, 38, 44**), and reference will be made to them at later points in this paper.

Investigation

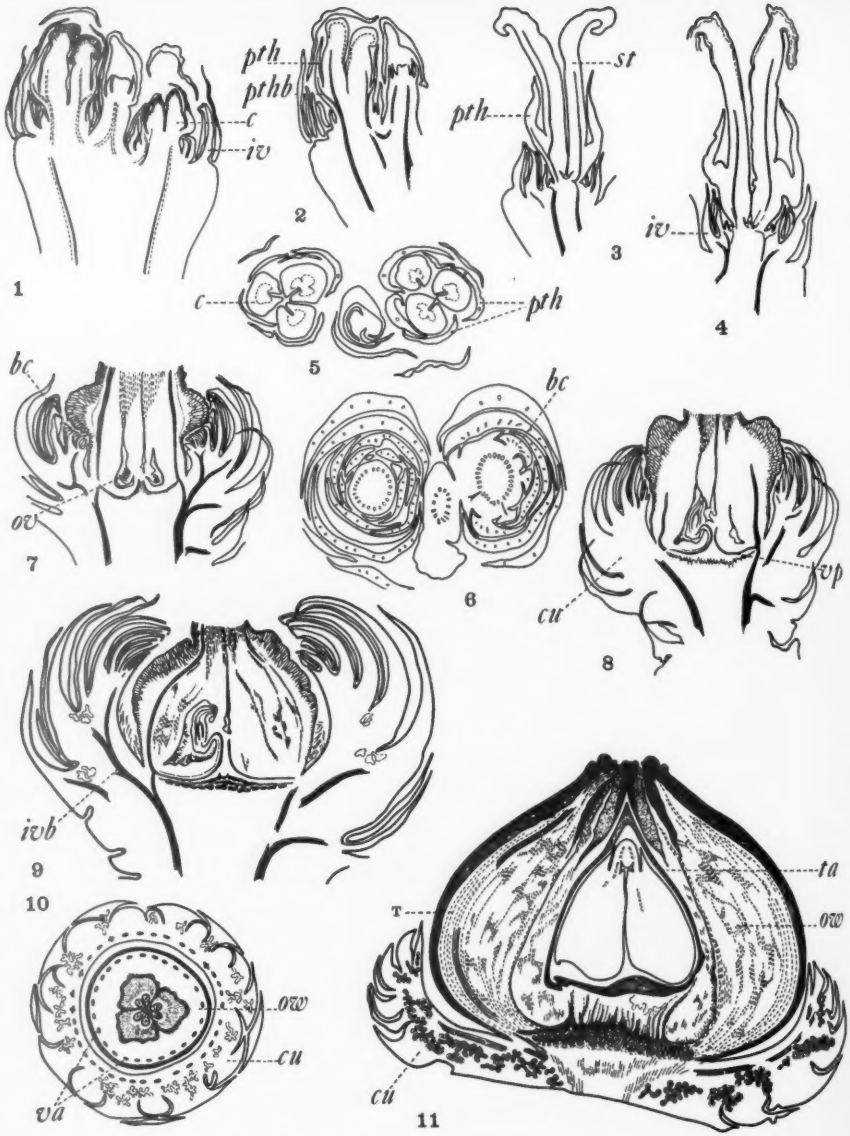
Two representatives each of the Fagaceae and the Juglandaceae—*Quercus rubra*, *Fagus americana*, *Carya glabra*, and *Juglans mandchurica*—have been selected for comparative treatments, and the conclusions are based upon an examination of serial sections of pistillate flowers and fruits at different developmental stages. Floral and embryological features of the Casuarinaceae will be dealt with in a later paper.

In the Fagaceae and Juglandaceae, collections of material for imbedding were taken at frequent intervals, from April 1 to early August, from trees on the campus and in the arboretum at Johns Hopkins University, and from the gardens of Dr. E. A. Andrews at Govans, Maryland. In all cases samples were secured from swelling buds and tips of growing shoots both before and following appearance of the pistillate flowers. Upon emergence of the young flower clusters, collections were made three to four times weekly to the time of pollination; after pollination daily for a week or two; and then at intervals of two to three days through June, July, and early August (periods of embryo and fruit development).

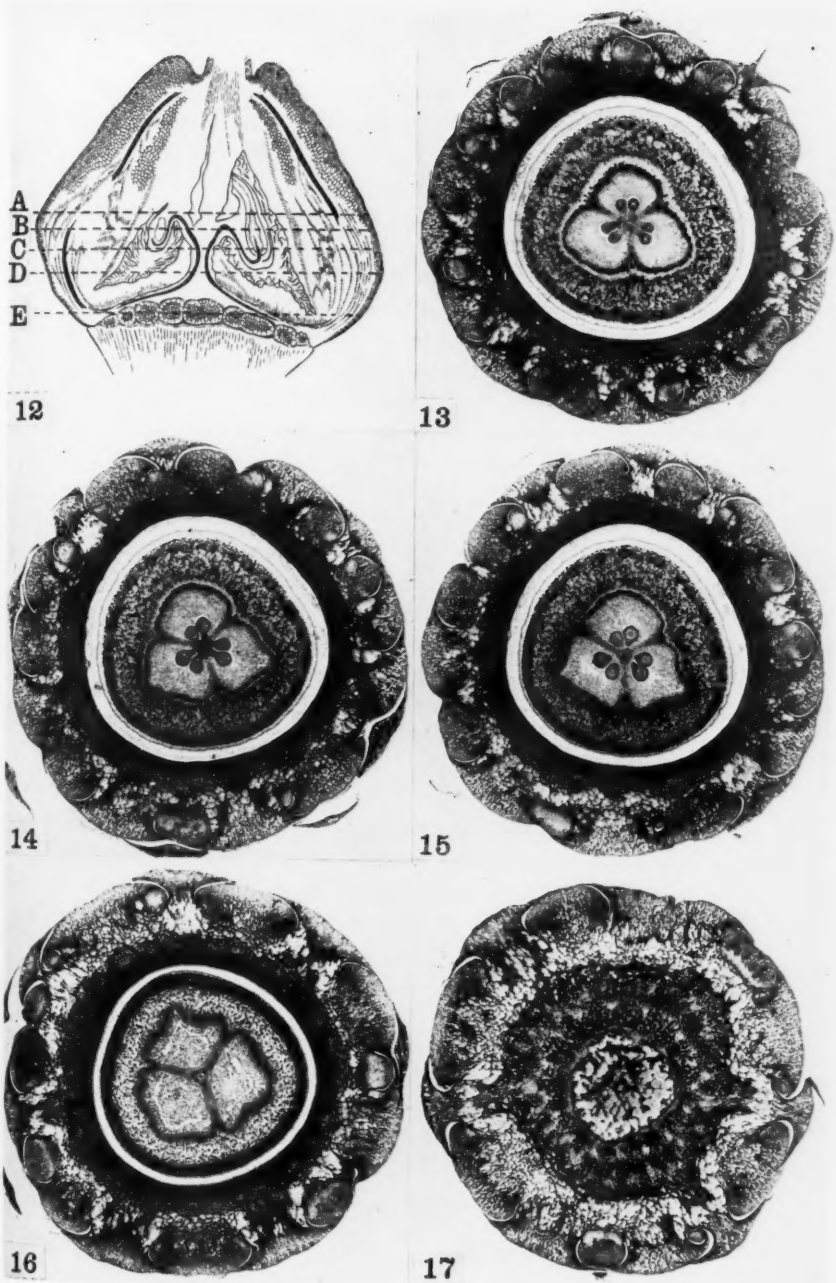
More than 500 fixations of inflorescences, flowers, and fruits were prepared and imbedded through three collecting seasons, and substantially the same technical procedure was followed as was reported in an earlier paper in this series (**27**). In preparing the fruits for paraffin imbedding, it was found advisable, after exhausting as much air as possible from the tissues, to substitute in dehydration mixtures of ethyl and n-butyl alcohols for the usual ethyl alcohol-xylene series (**45**). Serial sections 10–12 μ in thickness were secured with a Spencer sliding microtome (**26**). Only by this method has it been possible to procure the preparations required for these studies.

I. FAGACEAE

While the morphology and anatomy of the inflorescences and flowers of the Betulaceae have been studied more or less intensively



FIGS. 1–11.—*Quercus rubra*. Figs. 1–6, longi- and transections of young pistillate inflorescence and individual flowers, early to late May (1st season). Figs. 7, 8, young fruits, early to late May (2d season). Fig. 9, solitary fruit enveloped by bracteolate involucre. Fig. 10, transection of young fruit within cupule; distribution of vascular tissue both in cupular and floral axes shown. Fig. 11, partially matured fruit within cupule; testa of solitary seed incloses well developed embryo (*bc*, involucral scales; *c*, carpels; *cu*, cupule; *ivb*, involucral bundles; *iv*, involucre; *ov*, ovule; *pl*, placental axis; *pth*, perianth; *pthb*, perianth bundles; *st*, style; *ta*, testa; *fva*, vascular axis of ovary; *vp*, vascular supply to placenta; *ow*, ovary wall—axis+non-diverged pericarp).



FIGS. 12–17.—*Q. rubra*. Fig. 12, longisection of young fruit freed from enveloping cupule. Figs. 13–17, structure of fruit, including cupule, in transections at levels *A*, *B*, *C*, *D*, *E* of fig. 12.

by several investigators, including the recent detailed treatment of their inflorescence anatomy and morphology by ABBE (**1, 2**), similar studies involving the Fagaceae have been, since the early taxonomic and phylogenetic treatises (**18, 33, 36**), conspicuously meager. RENDLE (**34**) gives a more complete comparative account of generic differences in the family than are found in other recent phylogenetic studies of the flowering plants, but there is a noticeable deficiency in detail as well as scarcity of illustrations, with most of the latter taken from earlier works. BENSON'S contributions (**7**) were concerned chiefly with the megagametophyte in genera of the Betulaceae and Fagaceae; they added little to their floral morphology. Summarizing a comparative account of the archesporium, BENSON concluded that the Cupuliferae were less specialized than the Betulaceae, thus supporting EICHLER'S deductions based on floral morphology.

The Fagaceae generally are monoecious. The male flowers, in slender catkins or small capitate clusters, are borne usually in upper scale-leaf axils of the shoots of the current year; the female flowers, as solitary florets or 2- or 3-flowered cymose inflorescences within scaly, cuplike involucres, arise in the upper foliage leaf axils. Exceptions to this arrangement are found in the androgynous aments of *Pasania* and *Castanea*. From the transition region of the upper androgynous aments of the latter, a variety of inflorescence types have been reported (**18**), from 7-flowered staminate cymes with staminodia and pistil rudiment only in the primary florets to 3-flowered pistillate cymes all flowers of which are bisporangiate. In *Fagus*, staminate florets have been observed occasionally in the axils of upper leaves of the involucre of pistillate inflorescences, with the two normally oriented flowers of the inflorescence both bisporangiate (figs. 20, 32). Mixed inflorescences likewise are reported in genera of the Betulaceae (**2**), as well as the exceptional case of the replacement of individual flowers by miniature aments within the pistillate catkins of *Alnus*. Both in *Quercus* and *Fagus* the staminate florets not infrequently bear pistil rudiments.

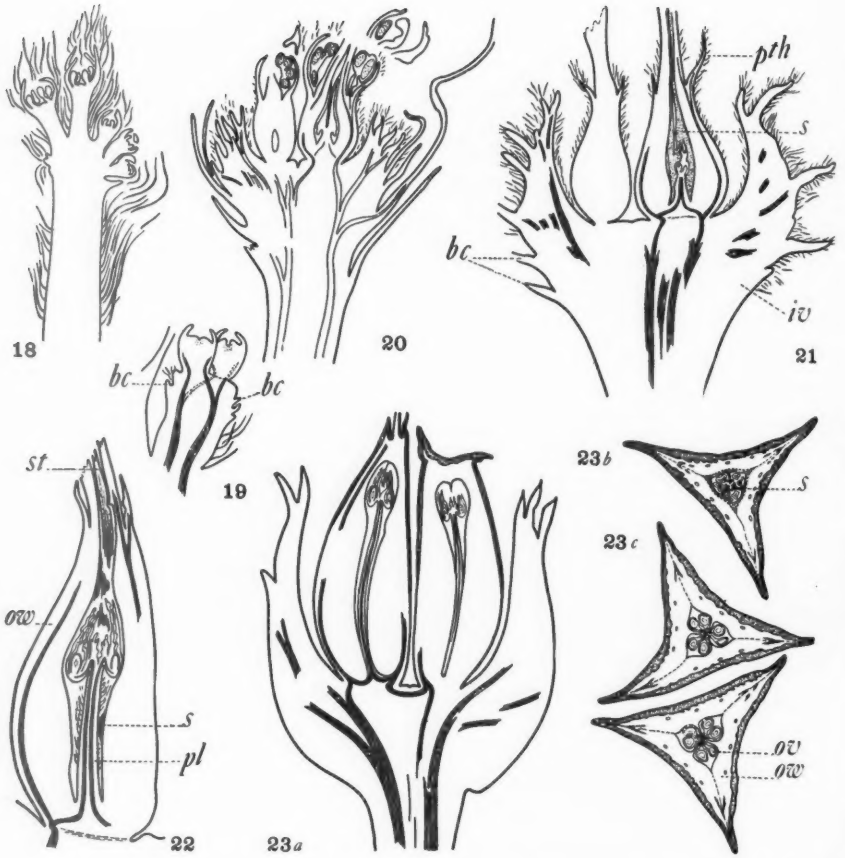
Familiar differences in the flowers and fruits of the six genera of this family arise from the various developments of the involucre and the number of flowers or fruits which it surrounds; for example, in

Castanea and *Fagus* four scale-bearing involucral segments inclose a 2- or 3-flowered inflorescence, while in the other four genera the female flowers (solitary or as 3-flowered cymes) are inclosed by a cupular scale-bearing involucre.

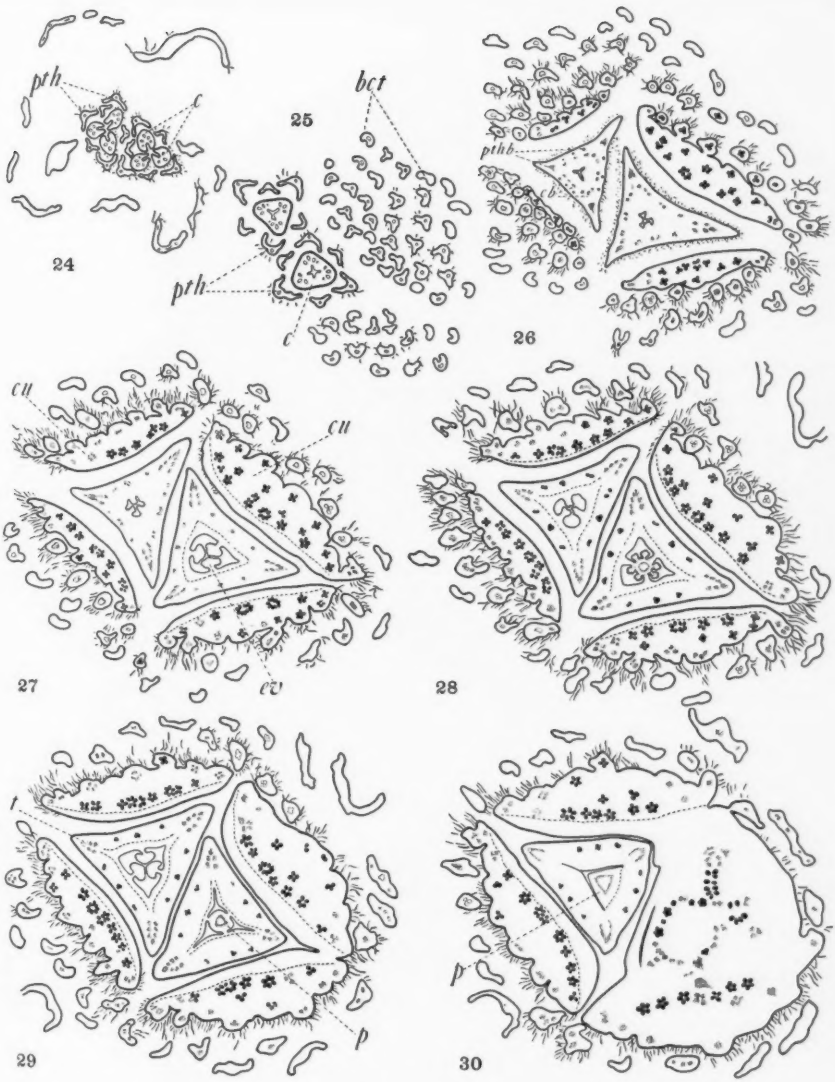
FLOWER AND FRUIT DEVELOPMENT IN THE BEECH.—Taxonomic accounts generally agree in describing the individual female flower as consisting of a 6-lobed calyx adnate to the 3-celled ovary, with as many styles as its cells, and one or two pendulous semi-anatropous ovules in each cell. These flowers, usually two together, are inclosed more or less completely by a 4-lobed, bracteolate involucre, the four basal bracts of which are longer than the involucral segments. Differences of opinion arise chiefly with regard to interpretation of the ovary and its placentation, and also as to the character of the involucre.

In the ontogeny of the pistillate inflorescence of *Fagus americana* the two florets are differentiated at the apex of the peduncle well in advance of development of the swollen bract-bearing portion of the axis beneath the flowers. In the young inflorescence illustrated in figure 19 both flowers already bear the primordia of perianth leaves and carpels, the latter appearing to arise in a slight depression of the receptacle and alternating with the inner set of perianth leaves. Rapid growth of the tissue underlying the three outer sets of floral members results in a fleshy, cupular perianth tube lined by the non-diverged carpellary tissue (figs. 20, 31, 33).

In the meantime, the portion of the floral axis inclosed by the carpels produces a short, thick columnal structure, at the tip of which ovule development is initiated. Centripetal growth of the incurved carpel margins takes place concurrently with development of the placental axis, and as cushion-like masses of parenchyma they envelop the placentae and ovules (fig. 21). Above the ovules and placental axis the carpellary ridges expand as parenchymatous wedges (figs. 27 and 29, *ev*) which meet in the center of the ovarian cavity but do not unite. Directly beneath the ovules the carpellary ridges grow conjointly with the placental axis (figs. 28, 29), thus forming the septa of the trilocular ovary. It is to be noted at this juncture that although carpellary tissue supports the placental column and closely invests the developing ovules, it does not bear the



FIGS. 18–23.—*Fagus americana*. Fig. 18, young staminate inflorescence showing median and one of lateral groups of florets. Fig. 19, young pistillate inflorescence. Fig. 20, pistillate inflorescence about April 15, consisting here of two bisporangiate and single staminate floret, latter appearing only in part. Fig. 21, young fruits inclosed by involucral segments. Fig. 22, single fruit showing vascular supply to placental axis in its relation to floral vascular axis, also to ovules. Fig. 23*a*, young fruits with investing involucral segments about June 15. Fig. 23*b*, *c*, transections of upper portion of two fruits illustrated in fig. 23*a*, *b*, above point of attachment of ovules, and *c*, at point of insertion of ovules on placental axis (*bc*, involucral bracts; *iv*, involucre; *pl*, placental axis; *pth*, perianth; *ov*, ovule; *ow*, ovary wall; *s*, septal tissue; *st*, styles).



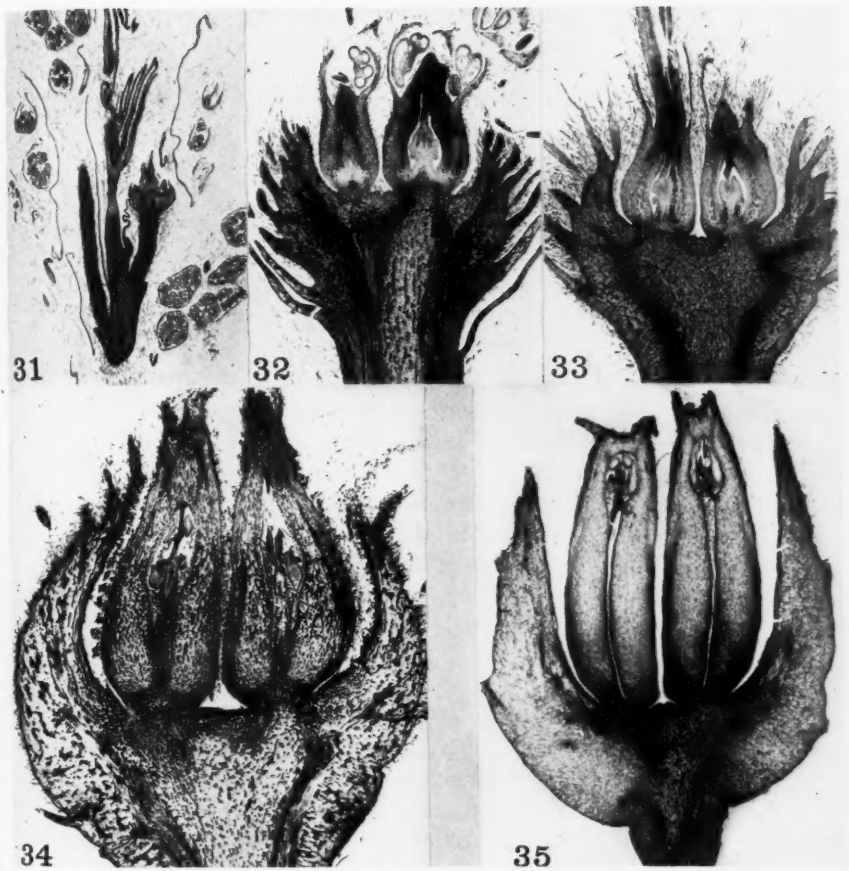
FIGS. 24–30.—*F. americana*. Transections of pistillate inflorescence at different levels from tip to base showing arrangement of vascular traces of florets and involucre and their connection with vascular axis of peduncle (*c*, carpels; *cb*, carpellary bundles; *cu*, cupule segments inclosing the two triangular florets; *bc*, involucral scales; *ev*, centripetally growing carpellary tissue; *ov*, ovules; *pth*, perianth; *pthb*, perianth bundles; *p*, placental axis). Dotted lines within florets differentiate axial tissue from inner carpellate portion of ovary wall. Shaded structures in center of right floret of fig. 28 are differentiating ovules.

ovules nor is it supplied with vascular tissue. Furthermore, with the inception of fruit development the carpellary ridges are withdrawn from the vicinity of ovules and placentae by expansion of the ovary wall (fig. 23*a*, *b*,), creating an incompletely triloculate condition in the upper part of the ovary.

The formative phases outlined in the preceding paragraph, excepting those of the last statement, take place before the opening of buds and emergence of the pistillate heads early in April. BENSON (7) likewise observed that in young female flowers of *Fagus sylvatica* examined early in April "ovules are already laid down, and the rudiments of the inner integuments seen to be forming." At anthesis (April 15–20) the state of ovary and placental development illustrated in figures 21 and 33 is reached. Subsequent development of the ovary wall and shaftlike placental axis just about keep pace, with a gradual shifting of meristematic activity toward the basal section of both; and by the mid-part of May the flowers present the structural features illustrated in figures 22 and 34. The ovules, now in an advanced state of development, have curved to an erect position, with micropyle and chalaza at right angles to the funiculus.

The manner of distribution of the vascular tissue to the ovary wall and placental axis is shown in figures 20–23, and as transections in figures 24–30. Each pistillate flower is supplied directly from the vascular axis of the peduncle with a vascular cylinder consisting of eighteen compound bundles, some distributed along the sides of the triangular floral axis while others are closely associated at the ridged angles of this axis. From each of these vascular units, strands to the axile placentae diverge at the base of the ovary. Within the placental column the placental bundles form a triangular vascular axis extending to the point of insertion of the ovules. In no case have they been observed to extend beyond that point. The perianth and stylar traces, as conjoint bundles, proceed from the units of the floral vascular axis through the marginal part of the ovary wall to the point of their divergence either to perianth or stylar members.

By the latter part of June or early July the young fruits of the beech, inclosed by spiny, coriaceous involucral segments, present the structural features illustrated in figures 23*a* and 35. Vestiges of



FIGS. 31–35.—*F. americana*. Fig. 31, section of young bud (early April) with outer scale leaves removed to reveal flower clusters, here consisting of one pistillate and portions of two staminate aments. Fig. 32, same type of inflorescence as in fig. 20. Fig. 33, typical 2-flowered pistillate inflorescence at pollination. Figs. 34, 35, young fruits invested by involucre.

perianth and stylar members appear as partially lignified appendages at the tips of the angular fruits. Lignification is evident also at the periphery of the fruit (nut), as also is the tomentose coating of the inner surface. The ovules suspended from the tip of the hairy placental axis with micropyles directed toward the apex of the ovarian cavity are found to be in various stages of abortion, only one, occasionally two, bearing normally enlarged megagametophytes.

Characterization of the pistillate flower of the Fagaceae, based on evidence furnished by ontogeny and anatomy, as an ovule-bearing axis closely invested by a cupular envelop, the latter the product of conjoint growth of stem and carpels, calls to mind conclusions reached by THOMAS (**38**) following a survey of the reproductive structures of the megaphyllous seed plants. He concluded that the flower commenced as a sorus or tuft of sporangia terminating a branch, this seed-bearing branch becoming more and more completely inclosed by a carpellate structure formed from the concrescence of cupules. The idea of the origin of seeds from a sorus-like structure in which only the central sporangium remains fertile has received support from paleobotanical researches, and is made the basis of an illustrated series by CHAMBERLAIN (**14**), who has depicted the hypothetical development of the seed condition in the Cycadofilicales from an assumed heterosporous fern ancestry. CHAMBERLAIN'S diagrams start with a sorus terminating a leaf, and progress through a series in the course of which the cupular indusium as well as the sporangium and megaspore walls become united to form the thick coat of the pteridosperm seed.

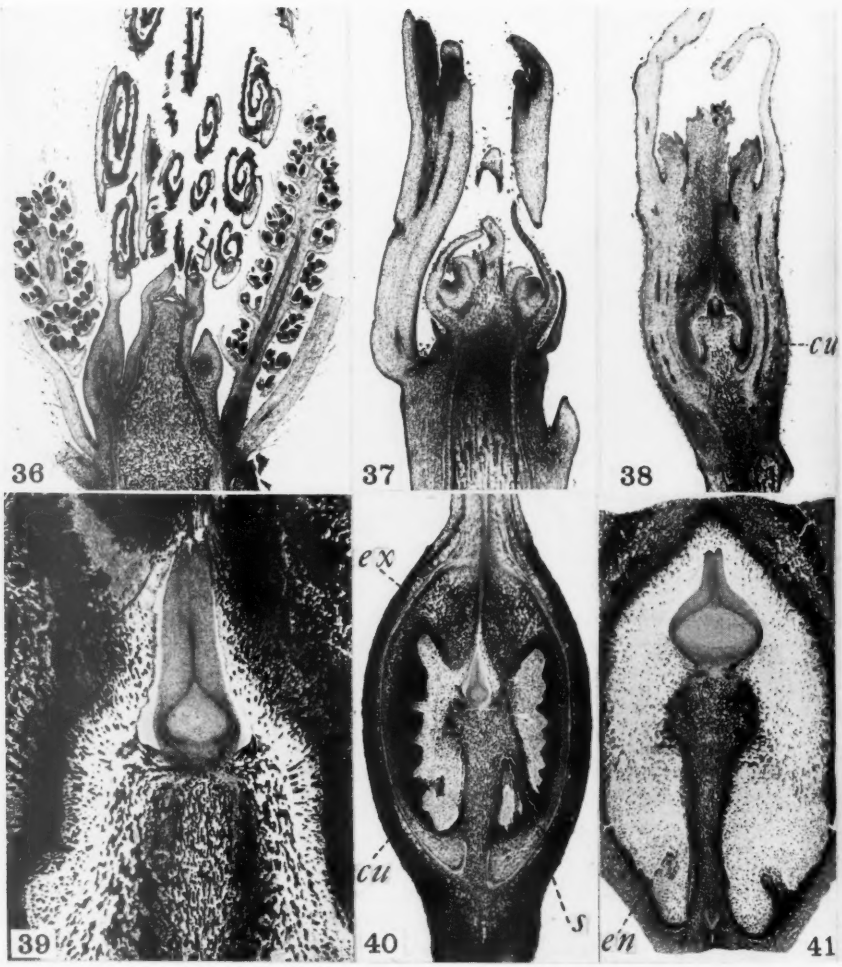
In supporting the theory of the evolution of the floral condition from a terminal sorus rather than from a condensed strobiloid body, THOMAS places special emphasis upon the aggregation of the sporangial structures at the tips of special branch systems.

PISTILLATE FLOWER AND FRUIT OF THE OAK.—The female flowers of *Quercus rubra*, each subtended by a bract and two bractlets, appear in short 2- or 3-flowered spikes from the upper axils of the leaves of the year. The individual flower, partially inclosed by a cupular involucre of imbricated scales, consists of a cup-shaped perianth tube non-diverged from the walls of an incompletely

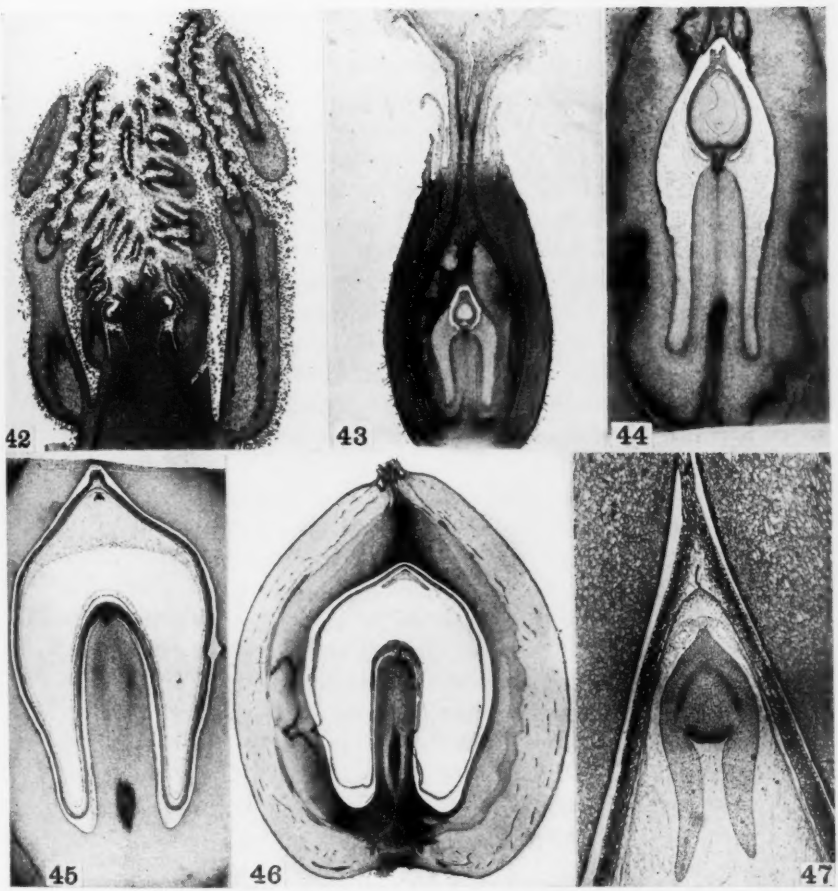
3-celled ovary, the latter bearing three erect styles stigmatic at their tips and along their inner surfaces. Development of the flower follows substantially the same sequence as that described in the beech. The character of the placentation and its origin are as observed in *Fagus*, but are somewhat more difficult to ascertain because of the more compact character of the placental axis and the prominent development of the centripetally growing ridges of carpellary tissue (figs. 4, 7, 8).

Anthesis and pollination in *Q. rubra* generally occur between May 7 and May 10 (figs. 1, 2, 3), but development of the placental tissue and formation of the ovules are not well advanced until the middle of June (fig. 4). At that time a strong development of procambium is evident in the vicinity of the placental axis, the strands differentiating along the base of the ovary to the placental column apparently as extensions of the floral vascular axis into the apical part of the flower. A single vascular ring of 20–24 bundles from which the vascular elements of both the perianth and the stylar members are diverged, as also those of the placental axis, proceeds from the base of the ovary through the axial portion of the ovary wall (figs. 6–10, 13–15). The branching of the perianth traces from this ring occurs slightly below the level from which the stylar traces are diverged.

From the latter part of July of the first season to early April of the second, only slight progress is made in the development either of ovules or of ovary. Only the bract-bearing involucral axis undergoes further development. As a fleshy cup-shaped structure, bearing at successively higher levels numerous scalelike leaves, it so completely incloses the flower through the fall and winter period that only the lignified vestiges of styles and perianth are distinguishable above its rim. By the early part of May of the second year (fig. 7), considerable expansion of the ovary wall is evident, as well as a deepening of the placental axis, and the ovules bear the rudiments of inner and outer integuments. A unique feature of the ovules, both of *Quercus* and *Fagus* but particularly marked in *Q. rubra* (and reported in the ovules of the magnolia), is the appearance in the elongated nucellus of a central several-layered strand of slender, procambium-like elements which extend through the base



FIGS. 36–41.—Pistillate flower and fruit of *Carya glabra*. Fig. 36, longisection of apical part of flower bud showing young staminate catkins; ovulate head as yet undifferentiate at apex of shoot. Fig. 37, young pistillate inflorescence; bracts of peduncle present. Fig. 38, median section of flower cut in plane of primary septum; ovule at archesporial stage. Fig. 39, ovule with mature megagametophyte. Fig. 40, median longisection of young fruit transverse to plane of primary septum, showing parenchymatous endocarp surrounding ovule and septum, also early differentiation of second septum which proceeds from two points —from margins of original septum and inner edge of carpel walls. Fig. 41, more advanced stage showing enlarged ovule at tip of shaftlike septum (*cu*, cupule; *en*, endocarp; *ex*, exocarp; s_2, secondary septum).



FIGS. 42–47.—*Juglans mandchurica*. Fig. 42, longisection of young pistillate inflorescence with enveloping foliage leaves. Fig. 43, median longisection of young fruit perpendicular to plane of septum, 5 days after pollination; pericarp clearly defined, also vascular tissue extending between involucral axis and ovary. Fig. 44, longisection of young fruit perpendicular to septum about 2 weeks after fertilization. Ovule shows megagametophyte with parietal layer of endosperm, also 18–20-celled embryo. Fig. 45, median section of nut perpendicular to septum 5 weeks after pollination; seed coat expanded to about full size, extending to all available space within the shell. Well defined layer of endosperm, cellular throughout, lines the seed coat, and embryo with cotyledons is distinguishable at apex of sac. Fig. 46, median longisection of nut closely invested by involucral axis (husk), sectioned perpendicular to primary septum. Fig. 47, enlargement of apical part of megagametophyte showing embryo as it appears sectioned in plane of carpels and primary septum.

of the nucellus and are continuous with the vascular tissues of raphe and funiculus. During the period of invasion and disorganization of the nucellar tissues by the megagametophyte, this procambial shaft is for some time a persistent and characteristic feature of the ovule. This and other features of the ovule and megagametophyte morphology of *Quercus* will be treated in greater detail in a later comparative embryological study of the Fagaceae.

The young fruit and one of the ovules as they appear at the time of fertilization, about June 16, are illustrated in figure 12. Development of the single seed and expansion and differentiation of the fruit coat are rapid from this stage, and by early July the fruit is half matured (fig. 11). In the developing wall of the fruit there is little if any distinction between the tissues of the pericarp and those of the conjoint stem (figs. 9–16). There is some lignification of the upper section of the pericarp, but a far greater amount of hardening takes place in the outer border of the axial section of the wall. The pericarp becomes desiccated and partially disorganized as seed development progresses (fig. 11).

Thus it appears that the fruits of the beech and oak meet the generally accepted definition of the "nut" type of fruit; that is, a hard, one-seeded, indehiscent fruit, usually produced from a compound ovary. Objection might be raised to this classification, however, on the grounds that these fruits develop from epigynous flowers, in which the hard outer rind is formed from non-diverged stem axis rather than from pericarp.

II. Juglandaceae

Rendle (**34**) has called attention to the resemblances between the Fagales and the Juglandales—in the catkin arrangement of the unisexual flowers; in the similarity in general plan of flower structure, including the presence in some genera of a pistil rudiment in the male flowers; and in the major role played by bracts and bracteoles in the protection and distribution of the fruit. Rendle believes that the epigynous perianth in the Fagales may be compared with the various stages of union of the perianth to the ovary in the Juglandales.

The pistillate catkins of the two species of the Juglandaceae

treated in this paper are solitary, terminal, and generally few-flowered; in *Juglans mandchurica* there are eight to ten in a spikelike inflorescence, while in *Carya glabra* there are but two or three (figs. 37, 42). Contrasting with the situation in *C. glabra*, where only two or three of the basal or first formed flowers of each inflorescence continue in development, is the situation in *J. mandchurica* where practically all the flowers in each ovulate head reach full development and are receptive at pollination. Furthermore, a large percentage of the flowers are pollinated and continue in fruit development (**27**). A comparative study of the inflorescence characters of the six genera and many of the species of the Juglandaceae has recently been made by MANNING (**28**), and an evolutionary series from primitive to advanced genera proposed on the basis of inflorescence types.

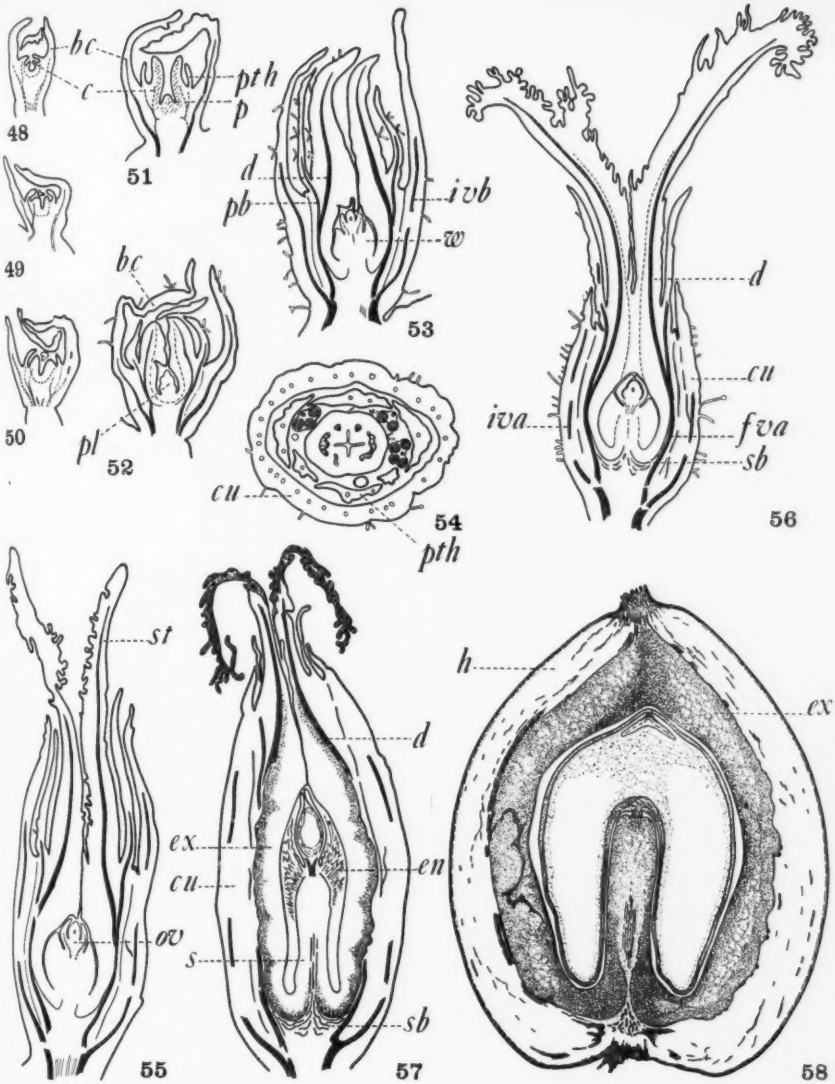
STRUCTURAL AND DEVELOPMENTAL FEATURES OF THE PISTILLATE FLOWERS OF JUGLANS AND CARYA COMPARED.—The female flower of *Juglans* and *Carya*, composed of a fleshy, 2-celled ovary (partly carpellate, partly axial in nature) crowned by an inconspicuous 1–4-leaved perianth and a pair of stigmatic styles, is inclosed by and united with a bracteolate, cupular involucre. Pistillate flowers of *Juglans* are generally described as bracted and 2-bracteolate with a 3–5-lobed (normally 4-lobed) calyx; those of *Carya* as bract fugacious, and calyx 4-lobed. According to other taxonomic statements, the female flower of *Carya* possesses a "perianth-like" involucre consisting of a bract and two bractlets in addition to one or two minute perianth leaves. In *Juglans* the involucre is united with the ovary to a little above the middle, while in *Carya* the involucral axis is united with the ovary to its apex.

The morphological character of the single orthotropous ovule of the Juglandaceae and its relation to the carpels have been discussed by several investigators, including DE CANDOLLE (**15**), VAN TIEGHEM (**43**), NAVASHIN (**29**), KARSTEN (**25**), NICOLOFF (**31**), BENSON and WELSFORD (**8**), and more recently by SHUHART (**37**) in a study of *Hicoria*. BENSON and WELSFORD'S conclusions supported those of VAN TIEGHEM that the vascular supply for each of the floral leaves is given off from the stem-stele at the base of the flower, and that the ovary is therefore superior; also that the ovule though appar-

ently developed from the top of the floral axis is really an outgrowth from the carpels, one or both of which contribute to its vascular supply. With the exception of VAN TIEGHEM and BENSON, the investigators mentioned have arrived at the conclusion that the ovule in the *Juglans* group is a development from the top of a floral axis in the formation of which the carpels have no part. Ontogenetic and vascular studies combined in the present investigation support this conclusion.

In the developmental account of the pistillate flowers of the Juglandaceae, *Juglans mandchurica* will be described in some detail, with briefer reference to comparable stages or features in the ovulate flowers of *Carya glabra*. Figures 42–47 and 48–58 show median vertical sections of the pistillate flowers and fruits of *J. mandchurica*, the collections dating from the early part of April to early June. Sections of young pistillate inflorescences collected early in April show flower primordia in various stages of differentiation (fig. 42). These primordia arise acropetally on a short peduncle, terminating the season's growth, until each inflorescence is composed of eight to ten buds. Figure 42 also illustrates a striking and characteristic feature of the young flower buds of *J. mandchurica*—the remarkable exhibition of hairs, glandular intermixed with long, stiff protective ones, which cover both surfaces of the unexpanded leaves, peduncle axis, and the flower primordia. The female inflorescence becomes visible above the leaves of the shoot early in May.

Three protuberances, one a little in advance of the other two, are the first structures to arise from the outer circumference of the broad tip of the flower primordium. These are the primordia of the involucral leaves, consisting of a single bract and two bracteoles. They reach prominent proportions with some elevation of the immediately underlying tissue before appearance of the four perianth lobes (fig. 42). At approximately the same time that the primordia of the perianth set commence their development, those of the carpels are initiated, the latter as crescentic ridges arising from the inner margin of the slightly concave floral axis. Figures 48–52 show successive stages from this point extending over a period of eight to ten days. From the appearance of the carpellary primordia, there is a noticeable growth not only in length of the perianth and carpel



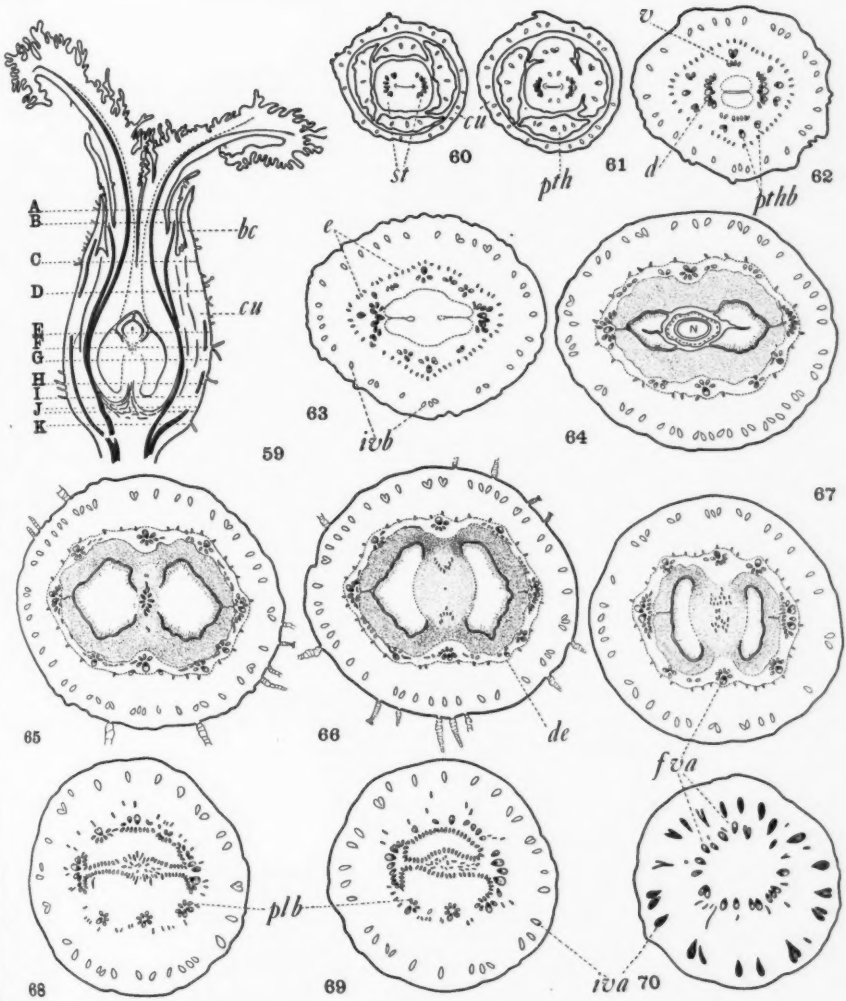
FIGS. 48–58.—Pistillate flowers and fruit of *J. mandchurica*. Figs. 48–53, median longisections of young pistillate flowers at different stages. Fig. 54, transection of female flower showing arrangement of parts, including staminodia. An interesting variation in another structure is here illustrated, in 2-, 3-, or 4-celled anthers all appearing in a floret. Fig. 55, flower at anthesis with ovule in megaspore stage. Fig. 56, flower at pollination time, ovule with young megagametophyte. Fig. 57, young fruit. Fig. 58, median longisection of partially matured nut, closely invested by fleshy involucral axis, same as in fig. 46 (*bc*, involucral bracts; *c*, carpels; *cu*, cupule; *d*, dorsal bundles of style and stigma; *ex*, exocarp (shell); *h*, husk (involucral axis); *fva*, bundles of floral vascular axis; *iva*, of vascular axis of involucre; *ov*, ovule; *pl*, placental axis; *pth*, perianth; *pthb*, perianth bundle; *sb*, septa bundles; *w*, winglike parenchymatous outgrowths from placental axis which completely fill loculi prior to fruit development).

lobes but also in the axial tissue underlying these floral parts, and coincident with this a broadening and deepening of the involucral axis. The simultaneous development of conjoined involucral and floral axes results in the elevation of a broad floral tube bearing on its rim involucral and perianth leaves, as well as stylar components. The line of union of floral and involucral axes is clearly distinguishable even from the earliest stages in floral development, and through the phases of maturation of the fruit is to be identified as a thin parenchymatous zone marking the line of dehiscence of involucral husk from the ovary wall (shell).

In the meanwhile, a revival of meristematic activity at the apex of the floral axis produces a conelike structure lying between and at the base of the carpels. Stages in the differentiation of the placental column and of the ovule primordium terminating this axis are illustrated in figures 51–53. Shortly before pollination in *Juglans mandchurica*, the single integument has about closed around the nucellus (fig. 55); at pollination ovule development is completed (fig. 56) and the nucellar region contains a young megagametophyte. Early in its development the expanding placental axis meets and apparently grows conjointly with the inturned margins of the two carpels, forming a vertical partition, in the plane of the carpels, which is partly axial and partly carpellary in origin.

Even in early stages of development of the placental axis, procambium can be seen to be differentiating toward the base of the placental column from each of the eight primary groups of bundles constituting the vascular cylinder of the flower, which is here distinguished from that of the involucre, the two sets connecting independently with the stem-stele (fig. 70). Continued growth of the floral axis below the point of divergence of the placental strands gives them at later developmental stages the appearance of diverging from the bundles of the floral axis at a point midway in the ovary wall (figs. 53, 55).

More advanced stages in floral development (figs. 55–56, 59–70) indicate that the placental bundles are divergent from those of the floral stele at a level slightly higher than that from which perianth and stylar bundles take their departure, and that they extend inward and—as reversely oriented bundles—downward to the base of



FIGS. 59–70.—*J. mandchurica*. Fig. 59, semi-diagrammatic longisection of mature pistillate flower showing vasculation. Figs. 60–70, structure in transverse sections at levels *A-K* of fig. 59 (*cu*, cupule; *d*, dorsal bundles to styles and stigmas; *de*, parenchymatous tissue at line of separation of ovary wall and involucral axis; *e*, small inversely oriented bundles at border of involucre; *fva*, bundles of floral vascular axis; *iva*, bundles of involucral axis; *plb*, small inversely oriented bundles derived from those of floral vascular cylinder and directed toward placental axis (*pth*, perianth; *pthb*, perianth traces; *st*, styles; *v*, lateral stylar bundles, many of which exhibit reversed orientation of elements).

the cup-shaped receptacle. At this point they curve toward the center, and as a thick cylinder of bundles, somewhat confused in orientation, extend upward into the placental axis (figs. 67, 68). Not far from the base of this axis they separate as two groups of bundles, one to either side of the primary septum (fig. 66), finally converging in the vascular supply to the single ovule (fig. 65). This distribution of the placental vascular tissue apparently to two placentae has been interpreted as suggesting an ancestral, 2-ovuled condition in the flowers of this group. VAN TIEGHEM (**43**) and BENSON (**8**) placed particular emphasis upon the evolutionary significance of this feature, and BENSON furnishes a series of diagrams of the female flower of *J. regia* intended to illustrate the greater development of one of the two so-called parietal placentae. A careful study of many series of sections of the pistillate flowers and fruits of *J. mandchurica* has failed to reveal any irregularity of development of the vascular tissue distributed to either side of the placental axis, and it is suggested that atypical cases may have been examined and illustrated in these earlier papers. Also the relationship between the cupular and perianth vascular supply illustrated in BENSON'S diagrams of *J. regia* bears a closer resemblance to the situation observed in *Carya* than that found in *J. mandchurica*.

Most of the space within the ovary of the walnut not occupied by the primary septum and the ovule is filled with soft parenchymatous tissue. Unlike that of the hickory (**27**), the packing tissue in the ovarian cavity of *Juglans* takes its origin as winglike outgrowths from the placental axis, developing at right angles to the plane of the primary septum (figs. 43, 53, 55, 56). Shortly before pollination, and for an interval of several days following, this tissue exists in a fairly compact state, completely filling the loculi and crowding close about the base of the ovule; but with growth of the ovule and later expansion of the seed coat, it is forced from the vicinity of the ovule, gradually becoming broken and chaffy in character (figs. 44, 57), except in areas of development of the secondary septa.

Developmental and anatomical features of the pistillate flower and fruit of *Carya*, essentially similar to those of *Hicoria pecan* described by SHUHART (**37**), show certain resemblances to and a few marked dfferences from those of the walnut. The resemblances are

found in the character of the ovary and its placentation, as also the manner of origin of the single orthotropous ovule. Such differences as occur are chiefly in vascular organization and evidently occasioned by reductions and compressions not found in the walnut.

Serial transections of young fruits of the hickory show outer and inner rings of vascular bundles within the fleshy involucral section, arranged in four segments corresponding to the four sectors of the cupular involucre. As in the pecan, four parenchymatous rays continuous with the edges of the involucral and perianth leaves separate the four segments of bundles, and are continuous with a zone of thin walled parenchyma marking the line of dehiscence of the cupular husk from the shell of the nut. The bundles of the inner ring of each of the four segments all show a reversed orientation of the vascular elements, and appear to be derived from the outer toral bundles by an incurving of these bundles at a point in the involucral axis slightly above the levels of divergence of the vascular elements to the perianth and involucral leaves. These reversely oriented bundles extend downward along the border of the ovary to the proximal portion of the cupule, at this point curving inward and then upward within the placental axis as two sets—one to either side of the primary septum. In *Carya*, the 3-leaved involucre and greatly reduced 1–2-leaved perianth are apparently supplied by a common, variously oriented stele, rather than by separate vascular axes as in *Juglans*. The vascular strands to the stigma tips of *Carya* are diverged from the middle inversely oriented ring of that stele, while the so-called dorsal bundles of the carpels are diverged from the center normally oriented ring. There are no vascular elements in the flower or fruit of *J. mandchurica* exactly corresponding to the dorsal carpel bundles in the hickory and pecan. The main bundles extending to the tips of the stigmas in *Juglans* are normally oriented and are diverged from those bundles of the floral vascular axis which border the midrib portion of each carpel.

Both in *Juglans* and *Carya* the sutures in the ovary wall corresponding to the lines of dehiscence of the shell bisect each carpel longitudinally. During maturation of the fruit of the walnut, lignification of the ovary wall commences with the inner section of the exocarp, extending thence to the outer border of the pericarp and

even to the axial portion of the ovary wall. Thus all eight sets of bundles constituting the floral vascular axis, as also the dorsal and lateral bundle groups to the stigmas, are included in the shell. This fact may not be apparent in figures 46 or 58, since they both depict early stages in the differentiation of the shell.

Summary

1. In both the Fagaceae and the Juglandaceae the ovary develops as a fleshy, urn-shaped tube (the product of conjoint growth of stem and carpels), bearing on its rim perianth and stylar components. A cauline placental axis growing conjointly with the inturned carpel margins produces the septa of an ovary, which is incompletely triloculate in the Fagaceae and biloculate in the Juglandaceae.

2. The involucre is cupular and consists largely of fleshy axis. In the Fagaceae the involucre incompletely incloses the ovary as a ringlike swelling beneath the flower, bearing numerous acropetally developed scales; in the Juglandaceae it completely incloses the ovary and is united with it, forming the fibrous-fleshy indehiscent husk of the walnut and the 4-valved dehiscent husk of the hickory nut.

3. The persistence in the Fagaceae-Juglandaceae series of inner and outer fleshy regions alternating with a stony layer in the seed-incasing body suggests a pattern well defined in the gymnosperms, even to the cupule-inclosed seeds of the pteridosperms, except that both protective and nutritive functions have been to a large extent transferred from a highly differentiated seed coat to the ovary wall and involucral structures. The outer fleshy layer (in the Fagaceae and Juglandaceae, involucral axis) may or may not be united with the stony section of the ovary, while the inner fleshy region (endocarp) eventually becomes dry and chaffy, and in the walnuts almost indistinguishable.

4. A marked reduction in the number of foliar appendages borne by the stem axis beneath or immediately adjacent to the flower as well as a closer association of such as do appear, in combination with other specialized characters (for example, reduced number of carpels

and ovules), point to a more advanced position for the Juglandaceae than for the Fagaceae.

5. On the other hand, fundamental resemblances in the floral structure of these families, including the catkin arrangement of unisexual flowers; the axial character of ovary wall and placentation; and the major role played by the involucral encasement of the flowers and fruits, argue in favor of their somewhat close alignment in any phylogenetic arrangement of the flowering plants.

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CONDUCTION OF RAINFALL BY PLANT STEMS IN A TROPICAL RAIN FOREST

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 505

PAUL D. VOTH

(WITH TWO FIGURES)

Introduction

Filmy ferns, bryophytes, and lichens are strikingly scarce on the trunks of many smooth-barked trees found on Barro Colorado Island, Canal Zone. This island is located in the Atlantic watershed of the isthmus in a tropical rain forest region characterized by an annual precipitation of more than 100 inches. The experiment reported here was conducted in and near the clearing occupied by the laboratory of the Institute for Research in Tropical America. A complete explanation of the paucity of epiphytes on some of the trees as compared with the abundance on others would necessitate a series of experiments before the role of various biotic as well as external environmental factors could be determined with accuracy. Time permitted the accumulation of data on only one of the possible factors involved, namely, the availability of water on the stems of woody plants during a portion of the so-called rainy season.

That a portion of every normal rain is prevented from reaching the soil by being intercepted, in the form of surface films and drops, by vegetation is well known. Some investigations on the interception of rain by foliage mention the precipitation which penetrates the canopy in reaching the ground, but do not report water flowing along branches and stems (**4, 11, 14, 17**). That some of the water, at least during heavier rains, may reach the ground by flowing down the trunks of trees is stated by BEALL (**1**), BURGER (**3**), MCMUNN (**10**), and SIMSON (**16**).

In 1870, NEY (**13**) began measurements of the water flowing down the stems of forest trees. In comparing two rains which amounted to 3.39 and 7.00 mm., NEY found, respectively, 4.8 and 20 per cent of the rain in the open flowing down stems. EBERMAYER (**5**) described the methods used by NEY. RIEGLER (**15**) reports that MATHIEU

used a receptacle large enough to collect water which flowed along the stem and all that penetrated the canopy. HORTON (**7**) calculated from RIEGLER'S (**15**) records that as much as 12 per cent of the total rain flows down the trunks of beeches while as little as 1.4 per cent reaches the ground along spruce stems; 1200 liters of water ran down a beech trunk during a rain of 77.5 mm. HOPPE (**6**) recorded the rainfall intercepted by rain gages placed under the canopy of forest trees in central Europe. By means of troughs placed around the trunks of trees in the same plot he recorded the amount of stem flow. Thus he was able to compute the amount of water actually retained by the tree crowns, which constitutes a loss to the forest soil. ZON (**19**, p. 26) states that in a conifer forest 0.7 to 3.0 per cent of the total precipitation reaches the ground along branches and trunks, but in a deciduous forest 15 per cent is conducted in this manner. MUNNS and SIMS (**12**) report that 1–5 per cent of the total precipitation reaches the ground by running down the stems of pine trees in the Appalachian region. BROOKS (**2**) recorded data on the rainfall distribution under *Cryptomeria japonica* as reported by OVSYANNIKOFF. The writer's calculations show that 1.6 to 14.6 per cent of the rainfall in the open may trickle down the trunks.

HORTON (**7**) diverted stem-flowing water into large containers by means of lead troughs fitted around the trunks of deciduous and hemlock trees in New York. The maximum amount collected from beech trees was 9.9 per cent of the total rainfall. He concludes that trees with rough bark conduct the least water to the ground. At present researches are being conducted on rainfall interception by trees in various parts of the United States.[1]

Except for the researches of RIEGLER (**15**), the results of the investigations just discussed are expressed in depth of water on the basis of crown area. The water returning to the ground along stems of plants on Barro Colorado Island has been calculated on the basis of depth of water in the cup and expressed as percentage of the total rainfall measured in the forest clearing.

[1] ROWE, P. B., ANDERSON, H. W., and DANIEL, T. W., U.S. Dept. of Agr. Forest Ser., Calif. Forest and Range Exp. Sta., Berkeley, Calif.

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FIG. 1.—Apparatus used in collecting water draining down stems of plants. *A:* leafless control stem (station I) with *Nothopanax guilfoylei* hedge and stations IV and V in middle left. *B:* upper smaller cup (station IV) and lower larger cup (station V) on *Nothopanax*.

Material and methods

A rain gage of the standard tipping bucket type, as described by KADEL (9), has been in operation on Barro Colorado Island for several years and served to determine the rainfall in the laboratory clearing for the period of this experiment. The electrically recorded data as well as direct measurements of the rainfall are a part of the scientific records of the island.

Plants utilized in this study were growing under cultivation or naturally near the laboratory. Only five stations with a total of five collecting cups were used, as follows:

A small unidentified tree was stripped of leaves and branches and placed in the ground at a location where the prevailing northeast winds would not be obstructed by buildings or vegetation. This 6-foot naked stem served as a control for the experiment (fig. 1*A*).

A young soursop or guanábana tree, *Annona muricata* L., growing under cultivation near the control station was selected because it was isolated except for the nearby canopy of the 75-foot trees at the edge of the clearing. This tree was in a vigorous vegetative condition and about 12 feet in height (fig. 2*A*).

A 9-foot high urticaceous tree, *Myriocarpa yzabalensis* (Donn. Smith) Killip, growing in the dense rain forest was selected for suitable trunk diameter and accessibility. Although this species is commonly considered a shrub, this specimen possessed a solitary trunk. The plant leaned from the vertical about 20°, and possessed few leaves. The latter condition is common for such understory plants (fig. 2*B*).

A single vertical branch of the shrub *Nothopanax guilfoylei* (Cogn. and Marchal) Merrill, an introduced species, supported two cups in tandem (fig. 1*A*, *B*). The ornamental hedge of which this plant was a part was trimmed about once a month and possessed terminal tufts of leaves. Occasionally a small group of compound leaves grew from short branches along the trunk. All stations were approximately 212 feet above sea level.

Rainwater flowing down the stems of these plants was collected in wide-angled disks or cups constructed of tin. The sides of the cups

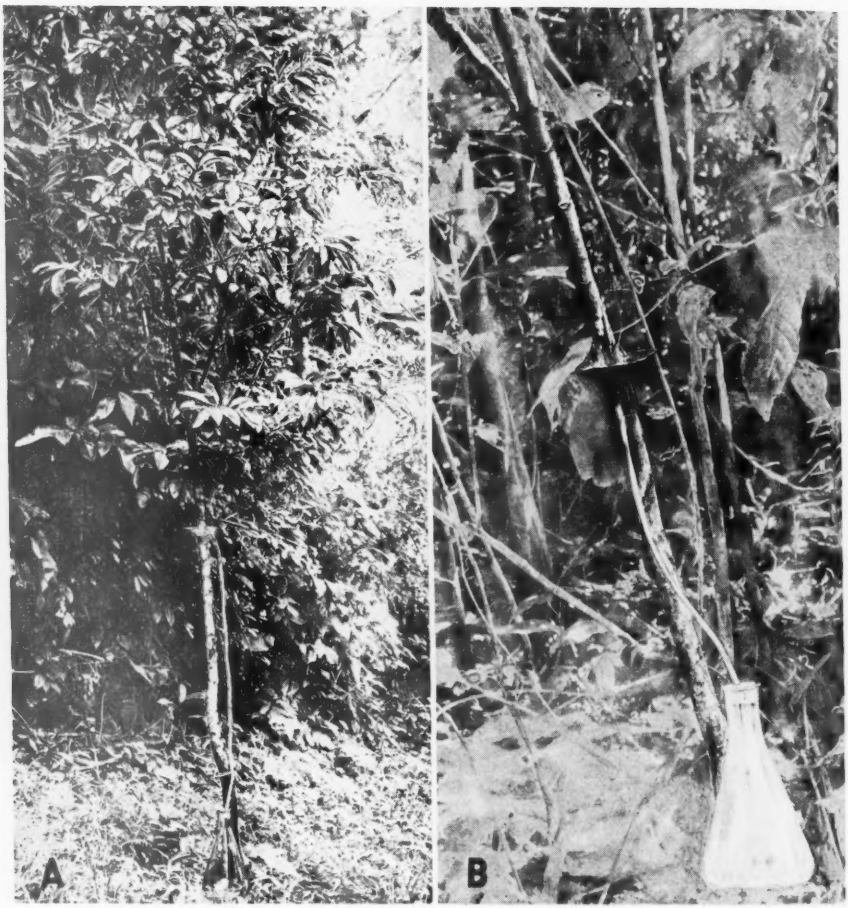

FIG. 2.—Apparatus used in collecting water flowing along stem of two tropical trees. *A: Annona muricata* in laboratory clearing with rain forest in background (station II). *B: Myriocarpa yzabalensis* growing in rain forest (station III).

formed an angle of about 65° with the sides of the stem. Cup dimensions are given in table 1.

A strip of rubber automobile inner tube, 2 inches wide and long enough to overlap slightly, was wrapped tightly around the plant at the desired height and the overlapping ends securely fastened with liquid rubber cement. Around this rubber collar the tin cup was modeled and soldered. Since all plant stems increase in circum-

TABLE 1

Station	Locality	Name of plant and total height (in.)	Circumference of stem at cup level (in.)	Cup			
				Height above ground (in.)	Circumference (in.)	Depth (in.)	Net area (sq. in.)
I.....	About 48 ft. from rain gage*	Leafless trunk of unknown species, 70	3.82	31	17.48	1.63	23.16
II.....	About 30 ft. beyond station I	Annona muricata, 144	3.94	50	17.48	1.63	23.08
III.....	About 70 ft. beyond station II	Myriocarpa yzabalensis, 112	3.82	24	17.48	1.63	23.16
IV.....	About 40 ft. from rain gage (near station I)	Nothopanax guilfoylei, 63	2.48	22.5	8.82	0.52	5.70
V.....	Same as station IV	Same as station IV	2.64	13	17.48	1.63	23.76

* Rain gage 12 feet from library.

ference downward, a downward pressure on the cup resulted in a tighter fit against the rubber cylinder and prevented leakage. As an added precaution against water loss, the inside of the cup and the stem immediately above it were coated with liquid rubber cement.

A 2-inch length of $\frac{1}{4}$-inch copper tubing was soldered into the side of each cup, approximately halfway between the rim and the base. Water accumulating in the cup rose to the level of the tube and ran down it and connecting rubber tubing into a suitable glass container. Insertion of the tube at some distance from the bottom of the cup prevented the daily accumulation of insects from obstructing the orifice. Such debris usually sank to the bottom of the cup. The

water in the container at the base of the plant as well as the water remaining in the cup was collected and measured every 12 hours, at 6 A.M. and 6 P.M.

Since the object of this experiment was to determine the relative amount of water available on the stem and branches as a possible factor in the development of epiphytes, water flowing down the stems was calculated in inches based on the net cup area. In table 2 such data are expressed in percentages of the rainfall measured by the rain gage.

Observations

The official rain gage was permanently installed in the laboratory clearing far enough from laboratory buildings and trees so that no interference was observable. Water collected in the cup on the control stem (station I) represents the accumulation of rain striking the cup directly and of water intercepted by the stem above the cup.

The *Annona* tree in the clearing possessed a well developed crown and was in full foliage. Repeated observations during storms showed that only an occasional drop of water filtered through the canopy and fell into the cup, which was placed on the trunk just below the divergence of the lowest branch from the trunk. Investigations by McMUNN (**10**) and HOPPE (**6,** p. 24) show that the crown of a tree permits the least penetration of water nearest the trunk. Since this *Annona* tree was very near the edge of the laboratory clearing, some water during a calm rain may have dripped from the 75-foot canopy of the rain forest trees, striking the foliage of the experimental tree. During rains accompanied by considerable wind the prevailing direction of the rain precluded such a dripping of water from the nearby forest. All water collected in the cup of station II, therefore, was that which ran down the leaves and along smaller branches until it finally flowed down the main stem.

The *Myriocarpa* tree in the rain forest near the laboratory possessed only a small number of leaves and was leaning from the vertical sufficiently so that most of the water flowing down its trunk was that which dripped from leaves and branches in the canopy of the main forest. It represents an average situation for many of the understory trees in such a forest where the canopy is 75–100 feet above the forest floor (station III).

The hedge plants of *Nothopanax* bordered an unobstructed path near the control stem (station I) and were nearer the official rain gage than the other experimental plants. Characteristically *Nothopanax* plants possess a main trunk only a few inches in length from which several nearly perpendicular branches originate. These branches are nearly leafless for a distance of about a yard and are repeatedly pruned at the top so that the terminal tuft of leaves originates from several axillary branches. A small cup was placed on a branch just under such a tuft of leaves so that very little rain could strike the cup directly. Most of the water accumulating in the cup, therefore, had been intercepted by the leaflets and had drained down the rachises, petioles, smaller branches, and the branch around which the cup was fastened (station IV).

A cup as large as those installed in stations I, II, and III was placed on the same *Nothopanax* stem about 9.5 inches below the smaller cup. Immediately above the large cup a short branch with three well developed compound leaves was growing from the vertical branch. Water accumulating in this cup had two origins: rain striking the cup on the windward side and water intercepted by a tuft of leaves lateral to the stem. This is designated as station V.

Only the volumes of water collected twice daily and the necessary dimensional data on the apparatus and plants utilized in the experiment were recorded on Barro Colorado Island. Rainfall in inches is recorded only for the rain gage. Records from all the experimental stations were converted into percentages of the officially recorded rainfall. The percentages are based on rainfall in inches of the net areas of the cups. Table 2 summarizes the results of the experiment.

The 14.01 inches of rainfall of August, 1938, was more nearly normal than the 19.36 inches of August, 1937, and approached to within 0.5 inch the amount recorded for August, 1936 (**18**). Of the 14.01 inches of rainfall recorded in August, 1938, 8.68 inches (62.0 per cent) fell in the 13 days covered in this experiment; therefore the amount of rain during this period was slightly above average. Table 2 gives the rainfall by 12-hour periods from August 5 to 18. During this period 1.70 inches fell in the night periods and 6.98 inches (80.4 per cent) in the daylight hours. Heavy rains taxed the capacity of the collecting containers, which at times were too small.

No records were taken when any doubt existed about the reliability of the measurements. With the high relative humidity of a tropical rain forest region it was possible to find traces of water in the cups after rains which yielded only 0.01 and 0.02 inches of water as measured by the official rain gage. While a rain of 0.04 inches was sufficient to cause an almost normal accumulation of water in the cups (August 7–8), NEY (13) reports that in European forests more than 2 mm. (0.08 inch) of rain must fall before water runs along the stems. At times evaporation losses were negligible because of the high relative humidity and the frequent and regular collection periods.

TABLE 2

AUGUST, 1938 (HALF-DAYS)	RAINFALL (IN.)	PERCENTAGES OF RAINFALL				
		I. CONTROL (BARE STEM)	II. ANNONA	III. MYRIOCARPA	NOTHOPANAX	
					IV. UPPER	V. LOWER
5–6	0.19	94.2	659.5	526.9	157.9	57.9
6	0.01	Trace	Trace	Trace	Trace	Trace
6–7	0.19	91.6	.....	798.9	95.8	61.0
7	0.54	102.4	563.1	.....	148.7	39.4
7–8	0.04	80.0	717.5	1027.5	280.0	32.5
8	0.16	96.9	388.1	512.5	86.9	61.3
8–9	0.41	99.0	622.2	573.9	348.5	24.1
9	0.22	92.7	310.5	682.7	330.9	33.6
9–10	0.13	101.5	1151.5	1103.1	411.5	30.0
10	1.71	89.2	.....	.....	289.2	26.7
10–11	0.32	102.2	1141.8	805.3	401.2	20.0
11–13	No rain for 5 half-days					
13–14	0.13	93.1	607.7	634.6	246.9	31.5
14	0.60	103.7	669.0	699.7	271.2	36.0
14–15	0.01	Trace	Trace	Trace	Trace	Trace
15	0.86	95.9	.....	.....	311.2	34.3
15–16	0.26	95.4	.....	.....	309.0	34.6
16	2.38	104.2	540.3	559.4	305.8	16.6
16–17	0.02	Trace	Trace	Trace	Trace	Trace
17	0.02	Trace	Trace	Trace	Trace	Trace
17–18	0.00	0	0	0	0	0
18	0.48	103.1	633.5	620.4	263.1	36.4
Average	.....	96.6	667.1	712.1	266.1	36.0

The cup on the leafless control stem collected nearly as much and even slightly more water than the rain gage. The lowest percentage (80 per cent for August 7–8) was recorded for a rain which fell at the

beginning of the collection period. The highest percentage (104.2 per cent for August 16) was recorded for the heaviest rain of the entire month. During a 2-hour period on August 16 a total of 2.21 inches of rain fell, which is 15.8 per cent of the total for the month. The average percentage of water collected by the control cup (96.6 per cent) in terms of the water collected by the rain gage is equal to the fidelity of rain gages placed some distance apart in areas of less rainfall (**8**).

Water accumulation in the cups on trees in stations II and III was very great. The least water collected from the *Annona* tree was 310.5 per cent (August 9) and from the *Myriocarpa* tree 512.5 per cent (August 8), while the most water collected from these trees was 1151.5 and 1103.1 per cent, respectively (August 9–10).

Most of the variations in the percentage of water collected at any one station may be attributed to differences in the intensity, direction, and time of the rain with reference to the collection periods. The maximum percentage of water collected by stations II and III was during the night periods (August 7–8, 9–10, and 10–11),when the total precipitation as recorded by the rain gage was relatively small (0.04, 0.13, and 0.32 inches, respectively). Rain during these periods fell either at the beginning of the collection period (6 P.M.) or at irregular intervals during the night, so that in either case a very high relative humidity was assured. During such periods water dripped from leaf to leaf and branch to branch, reducing the evaporation losses.

The average percentage for the *Annona* tree station of 667.1 approaches the 712.1 per cent average of the *Myriocarpa* tree station, even though the two stations were some distance apart, one in the open and the other in the rain forest itself.

The *Nothopanax* shrub (station IV), with a small terminal tuft of leaves, did not intercept and conduct via the stem nearly as much water as did the larger *Annona* tree or the canopy-covered *Myriocarpa* tree. With percentages of rainfall ranging from 86.9 to 411.5, it is evident that the maximum percentage recorded barely exceeds the minimum percentage intercepted and conducted by the *Annona* tree. Since the circumference of the cup at this station was much smaller than the others, rain striking it directly seems to be negli-

gible. The smaller percentage of water collected may be attributed to the small canopy of the plant.

The last (station V) was a large cup on the same *Nothopanax* stem and about 9.5 inches below station IV. Even though this cup was equal in size to the cups in stations I, II, and III, and was more exposed to direct rain than any other cup except the control, very little water accumulated. Other than direct collection of rain, the sources of water would be the 9.5 inches of stem between the upper and the lower cups and the three compound leaves growing from a small lateral branch immediately above this lower cup. The minimum percentage of water collected from station V was 16.6 of the total for that period and the maximum was 61.3. Both records are from daylight periods.

The leafless control stem was nearly devoid of lichens and possessed no other visible epiphytes.

The *Annona* and the *Myriocarpa* trees possessed a few non-fruiting crustose lichens and only scattered and poorly developed mats of clinging leafy hepatics.

Plants of *Nothopanax*, which are terminally pruned, often are infested with termites. The loose bark of such plants supports a luxuriant growth of leafy liverworts. Even where no termites have invaded the stem, large clusters of such hepatics grow where dripping water strikes a local area. These *Nothopanax* plants support a copious growth of leafy hepatics even though their habitat assures greater exposure and lower relative humidities during daylight hours.

Summary and conclusions

1. Water draining down stems of woody plants growing on Barro Colorado Island, Canal Zone, was collected by means of cups and measured. The amount of water collected was calculated to inches of water on the basis of the net area of the cup and expressed as percentage of the rainwater collected by the official rain gage. The experiment covered a period of 13 days in August, 1938.

2. A leafless control stem, equipped with a collecting cup, collected 80.0 to 104.2 per cent as much water as the rain gage.

3. Cups installed on the stems of *Annona* and *Myriocarpa* trees,

the former in the laboratory clearing and the latter in the rain forest, collected six to seven times more water than the rain gage.

4. Cups arranged in tandem on a stem of *Nothopanax* collected an amount of water consistent with their position. The smaller upper cup, which caught the water flowing down the leaves and smaller branches of the trimmed crown, collected an average of two and one-half times as much water as recorded by the rain gage. The lower larger cup, into which water from a short portion of the stem and from a lateral group of leaves drained, collected on the average slightly more than a third as much water as the rain gage. These *Nothopanax* plants possessed well developed mats of leafy liverworts and lichens.

5. From the results it seems conclusive that during the rainy season in a tropical rain forest area an amount of water flows down the stems of woody plants sufficient to support a copious growth of epiphytes such as lichens, liverworts, mosses, and filmy ferns.

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PHOTOPERIODIC RESPONSES OF SEVERAL VARIETIES OF SOYBEANS

H. A. BORTHWICK[1] AND M. W. PARKER[2]

(WITH TEN FIGURES)

Introduction

Previous work on the Biloxi soybean (**3, 8**) indicates that a succession of at least two relatively short photoperiods, each followed by a relatively long dark period, is necessary for photoperiodic induction in that plant. Such a succession of light and dark periods does not seem to be essential in all short-day plants, since in *Xanthium* (**5**) photoperiodic induction may follow exposure to a single relatively long dark period, whether the photoperiods are relatively long or short. The present studies report additional data concerning the photoperiodic response of several varieties of soybeans.

GARNER and ALLARD (**4**) have shown that soybean varieties planted during the summer months differ greatly in their time of bloom. They found that the variety Mandarin produced open flowers as quickly during the summer as during the winter months; other varieties, such as Biloxi and Peking, required many more weeks during the summer than during the winter. These differences, they concluded, were caused primarily by the different photoperiodic requirements of the several varieties.

From the work of GARNER and ALLARD it is evident that photoperiodic conditions appropriate to cause suppression of flowering in the Mandarin variety were not attained even during the summer when the length of effective photoperiod probably exceeded 15 hours. Since the range of photoperiods under which this plant would flower was obviously wide, it seemed possible that varieties might be found that could initiate flower primordia on long daily photoperiods or even in continuous illumination. Studies were made of Mandarin and several other varieties to determine their responses under extremely long photoperiods and under continuous illumination.

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For the studies dealing with floral initiation and growth, two experiments were carried out, one in the fall of 1938 and one in the spring of 1939, to determine the critical photoperiod for initiation in each of several varieties. Certain leaf areas were determined and stem-length measurements were taken. Data were also collected concerning flowering and fruiting under the various photoperiods employed. Two less extensive experiments, each involving a single variety, were carried out to obtain more detailed information concerning the influence of length of photoperiod upon the time and place of initiation of flower primordia. A chemical study was made of two varieties that differed greatly in their ability to initiate flower primordia under conditions of long photoperiod.

Material and methods

The varieties chosen for the experiments were selected on the basis of the average time required from planting to maturity when

TABLE 1

AVERAGE NUMBER OF DAYS FROM PLANTING TO MATURITY OF VARIETIES OF SOYBEANS

VARIETY	DAYS TO MATURITY*	VARIETY	DAYS TO MATURITY
Otootan	175	Ito San	105
Avoyelles	170	Minsoy	100
Biloxi	165	Wisconsin Early Black	100
Tokyo	140	Mandarin	100
Peking	125	Mc Crostie's Mandarin	
Mandell	110	Batorawka	
Hudson Manchu		Agate	90

* Data from MORSE and CARTTER (6).

grown under field conditions. They ranged in this requirement from 90 to 175 days (table 1), according to the data of MORSE and CARTTER (6).

Plants for all studies of floral induction were grown in 4-inch pots in the greenhouse. They were kept on warehouse trucks and were moved into adjoining dark chambers at the close of their respective photoperiods. The photoperiods for all lots receiving daily alternations of light and dark were begun at 8 A.M., and consisted of nat-

ural illumination extended with Mazda light of approximately 50 foot-candles for those lots receiving photoperiods longer than the natural ones.

In two experiments, twelve varieties of soybeans were each grown under nine different conditions of photoperiod. Six plants of each of the twelve varieties were placed on a truck and each truck was subjected to a different length of photoperiod. The photoperiods consisted of 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours daily. Photoperiodic treatment was begun when the plants first came through the soil and was continued without interruption until they were harvested.

In other experiments involving single varieties and two or three photoperiods, approximately fifty plants were used per treatment.

Experimental results

The twelve varieties used in the fall experiment of 1938 were the same, with one exception, as those used in the spring experiment of 1939. The variety Agate, used in the fall experiment, was replaced in the spring by Batorawka, an early maturing variety recently introduced from Poland. Since the other eleven varieties responded similarly in both experiments, the results of only the spring experiment are presented in detail.

This experiment was started March 21, and the plants were allowed to grow until May 9 before dissections were made. At this time all the varieties had bloomed and begun to set fruit on 8- and 10-hour photoperiods, and several varieties had produced visible buds or open flowers on still longer photoperiods.

The plants that had not yet produced open flowers or visible buds were dissected on May 9, 50 days after planting. At this time flower primordia were present on every plant subjected to continuous illumination in the seven varieties: Hudson Manchu, Mandell, Minsoy, Mc Crostie's Mandarin, Batorawka, Wisconsin Early Black, and Mandarin. These varieties are all early, requiring 110 days or less from planting to maturity. In Biloxi, Otootan, and Avoyelles, no flower primordia were present on plants receiving 16-hour or longer photoperiods. These varieties require 165 or more days to reach maturity, and behave as late varieties. In Tokyo and Peking va-

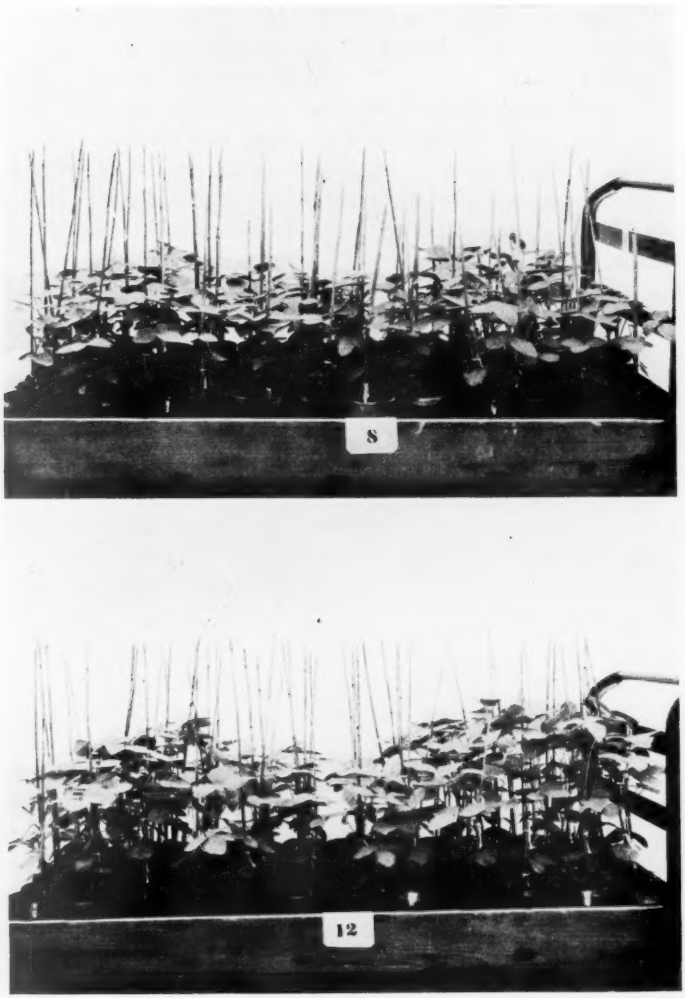

FIG. 1.—Twelve varieties of soybeans grown on 8- and 12-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

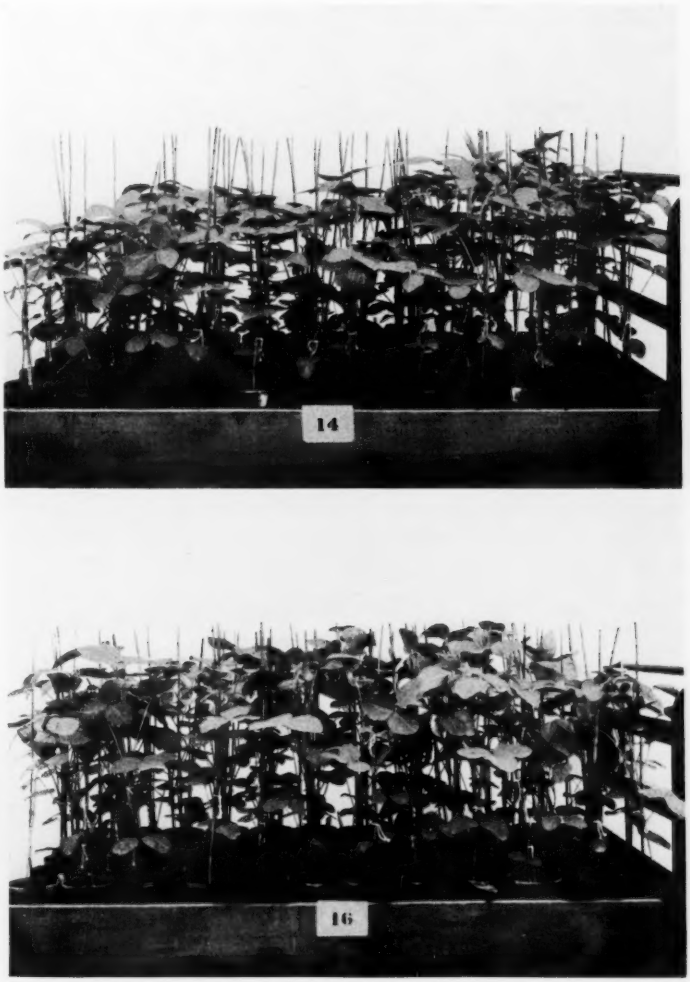

FIG. 2.—Twelve varieties of soybeans grown on 14- and 16-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

rieties, flower primordia were present in plants receiving 16-hour photoperiods. When these two varieties were subjected to photoperiods longer than 16 hours, flower primordia were formed on occasional plants of Tokyo but on no plants of Peking. These varieties are reported as intermediate in time of maturity, Peking requiring 125 days and Tokyo 140.

The results show that there is a relationship between earliness of maturity and length of photoperiod on which flower primordia can be initiated. In general, the earlier the variety matures the longer the photoperiod on which initiation can occur. This was probably to be expected from observations based on time of flowering as influenced by photoperiod (4). Since seven of the twelve varieties investigated were capable of initiating flower primordia with continuous illumination, this fact would seem to have a direct bearing on the problem of the relation of the dark period to photoperiodic induction in such short-day plants as soybeans. The fact that floral initiation occurred in several varieties regardless of the length of photoperiod employed might suggest that these varieties are not truly short-day plants, but should be regarded as indeterminate. While these varieties produced flower primordia on continuous illumination, blossoming and fruiting did not occur on photoperiods longer than 16 hours except in the variety Batorawka. In character and height of growth they also showed differences in response to photoperiod. In the past, macroscopic observations of flower bud formation, time of flowering and fruiting, and general growth behavior have been the criteria used in most cases in classifying the responses of plants to photoperiodic treatment. From this viewpoint these varieties should be regarded as short-day plants.

In all varieties the height attained by the plants was markedly influenced by length of photoperiod, greater height being associated with longer photoperiod (figs. 1–10). Part of this difference was caused by the fact that there were actually more internodes present in the plants that received long photoperiods than in those that received short ones. Plants grown on photoperiods shorter than the critical for flowering discontinued the differentiation of new structures at their tips rather early, while this was not the case in plants grown on photoperiods longer than the critical.

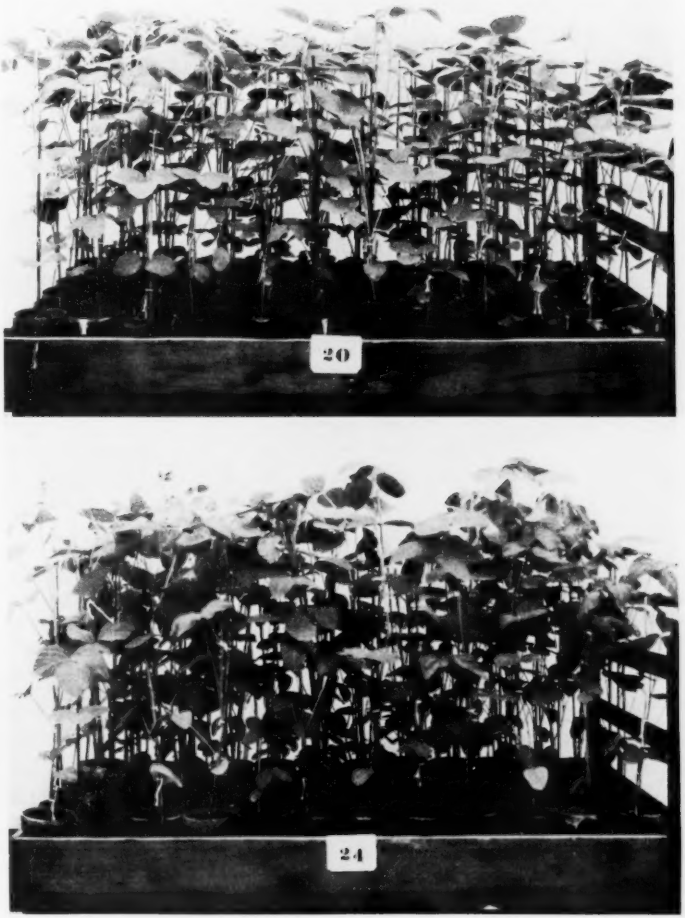

FIG. 3.—Twelve varieties of soybeans grown on 20- and 24-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

Part of the differences in plant height were also caused by differences in the lengths attained by corresponding internodes in response to photoperiodic treatments. Such differences appeared in internodes that reached maturity very early in the life of the plants. Thus the length of the first internode was greatest in lots receiving longest photoperiods.

TABLE 2

EFFECT OF 8-, 16-, AND 24-HOUR PHOTOPERIODS UPON STEM LENGTH AND LEAF AREA OF SOYBEANS. PLANTED MARCH 21; MEASURED MAY 4

VARIETY	MEAN LENGTH OF STEM FROM COTYLEDONS TO PRIMARY LEAVES (CM.)			MEAN AREA OF PRIMARY LEAVES (CM.^{2} PER LEAF)		
	8-HOUR	16-HOUR	24-HOUR	8-HOUR	16-HOUR	24-HOUR
Biloxi	3.9	5.2	7.0	24.2	24.6	25.5
Otootan	2.6	2.9	5.0	12.5	12.2	11.6
Avoyelles	2.7	3.8	5.7	14.7	13.4	19.2
Tokyo	4.7	4.8	7.9	24.9	27.3	27.7
Peking	2.2	2.6	3.2	10.9	13.6	14.0
Hudson Manchu	3.9	5.1	6.3	17.9	17.1	21.4
Mandell	3.4	4.2	5.0	17.2	24.1	22.7
Minsoy	3.5	4.0	5.1	9.1	9.1	9.5
Mc Crostie's Mandarin	3.9	5.2	6.7	15.9	19.4	19.5
Batorawka	4.4	5.3	7.5	23.5	27.6	33.1
Wisconsin Early Black	3.4	4.7	5.6	16.6	21.3	19.3
Mandarin	4.9	6.2	7.7	17.6	20.2	20.8
Mean	3.6	4.6	6.1	17.1	19.2	20.4

The mean areas of the primary leaves of each variety were also determined. These leaves were attaining their mature size at approximately the same time that elongation was occurring in the first internode. The differences in their areas as influenced by photoperiod were not so large nor so consistent as were the differences in internode length (table 2).

Although floral initiation in seven of the twelve varieties was not inhibited by long photoperiod nor by continuous light, the development of flowers and fruits showed response to photoperiod in all varieties.

The total number of pods 2 cm. or more in length was determined

for each lot of six plants when they were 50 days old (table 3). Plants of only one variety produced pods of this size on photoperiods of 18 or more hours. All the varieties that were able to initiate flower primordia in continuous light formed one or more pods on 16-hour photoperiod. On 14-hour photoperiod a few pods were formed on

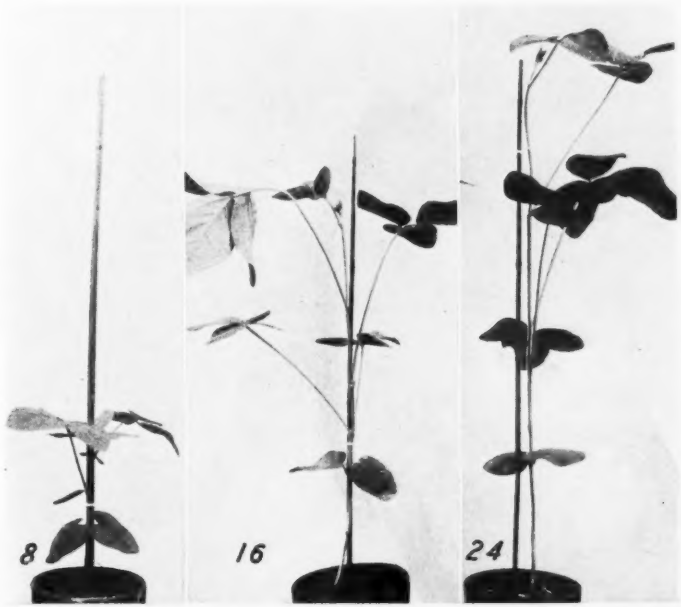

FIG. 4.—Biloxi soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

one additional variety, Peking, but none was formed on the remaining four varieties. On 12-hour and all shorter photoperiods there were one or more pods in every lot. Many pods shorter than 2 cm. were present when the data were obtained. These continued to grow and the number of large pods per lot increased. This increase occurred only in those lots of table 3 containing one or more pods and in the 14-hour lot of the variety Tokyo. The remaining lots showing no pods in table 3 did not develop any subsequently. From these re-

sults it seems evident that certain varieties can initiate flower primordia under conditions of long photoperiod at which their further development into mature fruits is inhibited.

The results obtained with Agate in the fall experiment of 1938 were similar to those obtained in the spring experiment with Batorawka. Agate plants produced visible buds and open flowers on every photoperiod used, including continuous light. Of the varieties

TABLE 3

EFFECT OF PHOTOPERIODS OF VARIOUS LENGTHS UPON PRODUCTION OF FLOWER PRIMORDIA, FLOWERS, AND FRUITS IN SOYBEANS. PLANTS 50 DAYS OLD

Variety	Total plants per lot of six bearing flowers or flower primordia						Total pods* produced per lot of six plants					
	14-hour	16-hour	18-hour	20-hour	22-hour	24-hour	8-hour	10-hour	12-hour	14-hour	16-hour	18-hour
Biloxi	6	0	0	0	0	0	28	22	1	0	0	0
Otootan	6	0	0	0	0	0	35	37	2	0	0	0
Avoyelles	6	0	0	0	0	0	40	48	16	0	0	0
Tokyo	6	6	3	0	1	1	35	36	22	0	0	0
Peking	6	6	0	0	0	0	25	32	30	3	0	0
Hudson Manchu	6	6	6	6	6	6	20	22	19	26	15	0
Mandell	6	6	6	6	5	6	29	18	9	19	1	0
Minsoy	6	6	6	6	6	6	29	35	29	30	8	0
Mc Crostie's Mandarin	6	6	6	6	6	6	21	24	31	26	24	0
Wisconsin Early Black	6	6	6	6	6	6	26	38	37	33	4	0
Mandarin	6	6	6	6	6	6	11	13	24	24	24	0
Batorawka	6	6	6	6	6	6	33	31	31	35	22	2

* Pods 2 cm. or more long.

tested, Agate and Batorawka seemed least inhibited in their floral development by long photoperiod. Since the effect of photoperiod on the time and place of origin of the first flower primordia was not determined in either of the previous two experiments, this point was studied further with the variety Agate.

Three lots of plants were started April 23, 1939. One was placed on 8-hour photoperiod, another on 16-hour, and the third on continuous illumination, before the seedlings began to emerge from the soil. On May 6, when the plants were thinned, dissections were made of some seedlings from each treatment. Flower primordia were

present on the 8-hour plants but not on the others. Since flower primordia were found on the 8-hour lot, the plants were harvested and dissected on May 8, 15 days after planting. At this time the largest plants were expanding their first compound leaf. Flower pri-

FIG. 5.—Ototan soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

mordia were present on forty-seven of the fifty-three plants harvested. The first flowers to differentiate were, with few exceptions, in the axil of the second compound leaf.

On May 12, 19 days after planting, the other two lots of plants were also dissected. Flower primordia were present on all the plants on 16-hour photoperiod and on all but a few stunted plants of the lot receiving continuous illumination. In the 16-hour lot the first flowers were formed in the axil of the fourth compound leaf in nearly

all the fifty plants dissected. In those receiving continuous light the first flowers were formed in the axil of the fifth compound leaf in all but a few plants, in which they were formed in the axil of the sixth compound leaf.

FIG. 6.—Tokyo soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

These observations on Agate soybeans show that long photoperiods cause a retarding influence upon time of initiation of flower primordia, even in a variety that promptly initiates them under 8-hour photoperiods. They were present on plants of the 8-hour lot

in less than 2 weeks after planting, but were not present at that time on plants grown on 16-hour photoperiod or on continuous light.

The differences in position of the lowermost, and therefore first formed, flower primordia on plants of the three lots also showed that photoperiod influenced the time, and consequently the place, of origin of flower primordia. The photoperiodic stimulation resulting

FIG. 7.—Peking soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

from treatment with 8-hour photoperiod was sufficient to bring about formation of flower primordia in the bud in the axil of the second compound leaf. This same bud in the other two lots developed into a vegetative structure, however, as did also the next one above it. Evidently the photoperiodic stimulation resulting from the other two treatments was not of sufficient intensity to bring about flower bud formation when the buds in the axils of the second and third compound leaves were still in condition to be influenced

in their development. A difference also occurred in the positions of the first flowers in the lots on 16-hour and continuous photoperiod. These results show that as the photoperiod is increased the length

FIG. 8.—Batorawka soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

of time necessary for the initiation of flower primordia is also increased.

Somewhat similar responses have also been obtained with other varieties. The variety Ito San was grown on 14- and 18-hour photo-

periods from the time of emergence of the seedlings from the soil until the plants were harvested. The first flower primordia on the 14-hour lot were in the axil of the fourth compound leaf of most plants and in the axil of the third in the remaining ones. On the

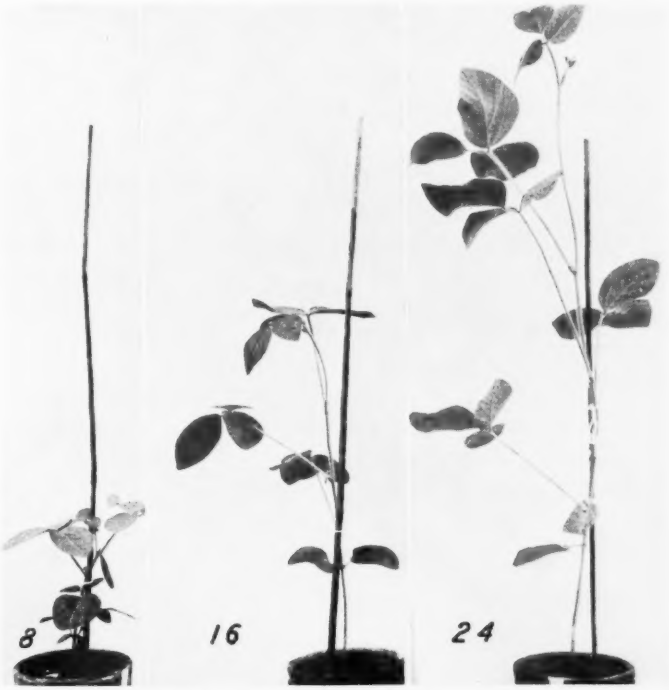

FIG. 9.—Mandarin soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

18-hour photoperiod the first flower primordia were in the axil of the twelfth leaf in most plants and in the axil of the thirteenth in the rest. The same type of response has also been observed in Biloxi. When grown on 8-hour photoperiod these plants initiate their first flower primordia in the axil of the second compound leaf, but when grown on photoperiods of 16 or more hours they initiate their first

flower primordia in the axil of the thirtieth leaf or at even higher positions. In all experiments in which Biloxi soybeans have been

FIG. 10.—Biloxi and Agate soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Upper row: Biloxi; lower row: Agate. Photographed October 7, 1938, 31 days after planting.

grown for several months on photoperiods of 16 hours or more, the plants have ultimately initiated flower primordia.

The data concerning time and place of origin of first flower primordia in these different varieties may mean (1) that there is a slow

accumulation of a rather weak photoperiodic stimulus received from long photoperiods, or (2) that the plant becomes progressively more responsive to the same degree of stimulation as it grows older. Data supporting this last view have been published previously (**2**). No data have been obtained either for or against the other assumption.

Chemical studies of Biloxi and Agate soybeans

It has been shown that Biloxi soybeans remained vegetative and Agate soybeans promptly initiated flower primordia when grown on a 16-hour photoperiod. Since the two varieties made these different responses when grown under identical environmental conditions, they presented an opportunity to compare the metabolism of vegetative and reproductive plants of the same species that had received the same amount of total radiation.

For this experiment Biloxi and Agate seeds were planted in white quartz sand in four different control rooms. The planting was arranged so that alternate pots contained Biloxi seeds and the remaining pots Agate seeds. The pots were flushed daily with a four-salt nutrient solution which from previous experience gave good growth in the control rooms employed. Light was furnished by carbon arc lamps burning Sunshine carbons. The arc light was supplemented by Mazda light. The intensity of the carbon arc lights was approximately 1300 foot-candles in two rooms and 1800 foot-candles in the other two rooms. The intensity of the Mazda light used was 160 foot-candles in all rooms. The temperature was maintained at 70° F. $\pm$ 2° in all four rooms.

The plants were thinned to four per pot 10 days after planting. When the plants were 3 weeks old a composite sample of each variety was taken from all four rooms by collecting one plant from each pot. This procedure was repeated at the end of 4, 5, and 6 weeks, respectively. The plants used for the 2-week samples were grown in the same rooms under the same conditions, but since the plants were very small at 2 weeks, all of them were used for this sample. When harvested, the plants were cut at the cotyledonary node and the leaves and stems were separated. Twenty or more plants from every sample taken were examined morphologically.

No flower primordia were present on any Biloxi plants during the

entire experiment. In Agate there were no flower primordia at the end of the second week, but at the end of the third they were observed on eleven of twenty-four plants examined. At the end of the fourth and all subsequent weeks they were present on all Agate plants.

During the first 4 weeks the Agate plants differentiated new leaf primordia at the apex of the main stem more rapidly than did Biloxi. At the age of 2 weeks Biloxi had an average of 10.0 nodes per plant and Agate 10.3. At the end of 4 weeks Biloxi had 15.6 nodes and Agate 17.7. By the end of the fifth week terminal inflorescences had been formed in Agate plants. With the advent of these, differentiation of further nodes and internodes in the main stems ceased. The Biloxi plants continued to form new leaf primordia and at the end of 6 weeks had an average of 22.1 nodes per plant.

Throughout the entire period of the experiment the Biloxi plants were erect and sturdy with no evidence of branching. On the other hand the Agate plants had stems of smaller diameter, showed a tendency toward twining, and developed vigorous side branches before the end of the experiment.

The chemical methods, except those used for the determination of amide and nitrate nitrogen, have been reported previously (**7**). Amide nitrogen was determined in duplicate 50-ml. aliquots of the extract after they had been hydrolyzed with 3 ml. of concentrated sulphuric acid for 2.5 hours under reflex condensers. After hydrolysis the solutions were nearly neutralized with sodium hydroxide and the ammonia was determined by aeration as reported (**7**). The nitrate nitrogen method employed was the same as used previously (**7**), except that the determinations were made on the extracts after the amide nitrogen had been removed.

The results of the chemical analyses of Biloxi and Agate soybeans are shown in tables 4, 5, and 6. The percentage of dry weight in the leaves of Agate plants was greater than in the leaves of Biloxi throughout the experiment, and in the stems the same condition existed except at the end of the sixth week. Agate plants accumulated dry weight at a more rapid rate than Biloxi for the first 5 weeks, and this accumulation occurred in both leaves and stems. During the sixth week the Biloxi plants accumulated dry weight

much more rapidly than in previous weeks and at the end of the experiment exceeded the Agate plants in dry weight.

Results of the nitrogen analyses are shown in table 5. The percentage of total nitrogen in both varieties decreased with age in both leaves and stems. The leaves of the Biloxi plants had a slightly higher percentage of total nitrogen than the Agate leaves, throughout the experiment. In the stems the percentage of total nitrogen

TABLE 4

PERCENTAGE MOISTURE AND DRY WEIGHT; AND FRESH AND DRY WEIGHT PER PLANT OF BILOXI AND AGATE SOYBEANS AT VARIOUS AGES

Variety	Age (weeks)	Percentage moisture		Percentage dry weight		Fresh weight per plant (gm.)			Dry weight per plant (gm.)		
		Leaves	Stems	Leaves	Stems	Leaves and stems	Leaves	Stems	Leaves and stems	Leaves	Stems
Biloxi...	2	85.91	91.63	14.09	8.37	1.45	0.80	0.65	0.16	0.11	0.05
Agate...	2	85.49	91.16	14.51	8.84	1.42	0.66	0.76	0.16	0.09	0.07
Biloxi...	3	84.48	88.77	15.52	11.23	4.65	2.25	2.40	0.62	0.27	0.35
Agate...	3	83.65	88.49	16.35	11.51	4.48	2.38	2.10	0.63	0.24	0.39
Biloxi...	4	86.37	88.79	13.63	11.21	10.78	6.88	3.90	1.38	0.44	0.94
Agate...	4	85.02	87.43	14.98	12.57	11.21	6.13	5.08	1.56	0.64	0.92
Biloxi...	5	86.81	87.95	13.99	12.05	19.44	12.08	7.36	2.58	0.89	1.69
Agate...	5	84.90	85.73	15.10	14.27	22.06	12.17	9.89	3.54	1.41	2.13
Biloxi...	6	84.74	84.69	15.26	15.31	44.79	28.96	15.83	6.84	2.42	4.42
Agate...	6	84.29	85.17	15.71	14.83	42.17	22.65	19.52	6.45	2.89	3.56

in Biloxi was greater than that in Agate at ages of 3, 4, and 5 weeks. The greatest difference in the percentage of total nitrogen in both leaves and stems occurred when the plants were 4 weeks old. At this time all Agate plants were differentiating flower primordia while the Biloxi were strictly vegetative.

The percentage of soluble non-protein nitrogen in the leaves was approximately the same in both varieties at 2 and 6 weeks, but in the stems was greater in Agate than in Biloxi. When the plants were 3, 4, and 5 weeks old the Agate leaves and stems contained a lower

TABLE 5

NITROGEN DISTRIBUTION IN LEAVES AND STEMS OF BILOXI AND AGATE SOYBEANS AT VARIOUS AGES
RESULTS BASED ON PERCENTAGE DRY WEIGHT

VARIETY	AGE (WEEKS)	TOTAL NITROGEN		SOLUBLE NON-PROTEIN NITROGEN		AMINO NITROGEN		AMIDE NITROGEN		AMMONIA NITROGEN		NITRATE NITROGEN	
		LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
Biloxi	2	6.884	5.675	1.675	3.835	0.468	1.565	0.071	0.621	0.014	0.024	0.184	0.370
Agate	2	6.671	5.848	1.626	4.423	0.461	1.832	0.062	0.679	0.014	0.023	0.331	0.520
Biloxi	3	5.573	3.357	1.263	1.897	0.580	0.757	0.045	0.276	0.019	0.009	.....	.....
Agate	3	5.284	3.214	1.144	1.755	0.459	0.669	0.030	0.226	0.012	0.009	.....	.....
Biloxi	4	5.473	3.060	1.467	1.766	0.528	0.642	0.044	0.134	0.007	0.009	0.029	0.036
Agate	4	4.766	2.434	1.055	1.241	0.347	0.549	0.020	0.095	0.007	0.000	0.020	0.040
Biloxi	5	4.896	2.733	1.365	1.610	0.479	0.722	0.064	0.216	0.014	0.008	0.129	0.191
Agate	5	4.424	2.004	0.962	1.044	0.450	0.511	0.046	0.112	0.020	0.014	0.060	0.105
Biloxi	6	4.463	2.012	1.219	1.052	0.446	0.575	0.131	0.248	0.020	0.013	0.111	0.104
Agate	6	4.347	2.158	1.216	1.268	0.465	0.519	0.108	0.256	0.013	0.007	0.121	0.195

percentage of soluble non-protein nitrogen than the Biloxi leaves and stems.

The percentage of amino nitrogen varied in both leaves and stems in the same manner as the soluble non-protein nitrogen. Amide nitrogen in the leaves of Biloxi plants was always higher than in Agate leaves, and was also higher in Biloxi stems at 3, 4, and 5 weeks than in Agate stems.

The percentage of ammonia nitrogen in the two varieties was not greatly different during the experimental period and the slight differences show no particular trend. The amount of nitrate nitrogen was much higher in the Agate than in the Biloxi plants at 2 weeks. In later samples the differences were not great and showed no particular trend.

In general the nitrogen content of the two varieties was not greatly different. Such differences as occurred were greatest when the plants were 3 and 4 weeks old and it was at this time that floral initiation started to occur in the Agate variety.

Results of the carbohydrate analyses are shown in table 6. The percentage of reducing sugars in the leaves of the Agate plants was greater than in Biloxi at 3, 4, and 5 weeks. The percentage of reducing sugar in the stems was higher in Agate than in Biloxi in all except the 6-weeks sample. Differences in relative amount of total sugars in the two varieties were similar to differences in reducing sugars. The percentage of sucrose in the leaves did not differ greatly in the two varieties until the fourth week. At this time the Agate leaves contained much less sucrose than the Biloxi, but the stems contained more.

The percentage of starch in the Agate leaves was much greater than in the Biloxi leaves until the sixth week. The maximum amount was found in Agate at 3 weeks and in Biloxi at 6 weeks. In the stems the differences in percentage of starch in the two varieties were not so great and a maximum percentage occurred in both at 3 weeks.

The total carbohydrate content of Agate reached a maximum when the plants were 3 weeks old. In older plants there was a progressive decrease. On the other hand, in Biloxi the highest total carbohydrate content during the experiment occurred when the plants were 6 weeks old.

Since it is not known whether this difference in the rate of carbohydrate accumulation exists in all varieties that initiate flower primordia on any photoperiod, no general conclusions can be drawn. Nevertheless maximum total carbohydrates occurred in the leaves of Agate plants at the time floral initiation was beginning. While

TABLE 6

CARBOHYDRATE CONTENT OF LEAVES AND STEMS OF BILOXI AND AGATE SOYBEANS AT VARIOUS AGES. RESULTS BASED ON PERCENTAGE DRY WEIGHT

VARIETY	AGE (WEEKS)	REDUCING SUGARS		TOTAL SUGARS		SUCROSE		STARCH	
		LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
Biloxi	2	1.228	1.649	1.937	1.995	0.575	0.179	2.051	1.242
Agate	2	1.096	2.012	1.833	2.489	0.606	0.283	4.225	1.663
Biloxi	3	2.133	3.277	3.106	4.265	0.792	0.748	4.542	2.315
Agate	3	2.538	4.327	3.553	5.334	0.819	0.756	9.229	2.163
Biloxi	4	2.825	3.336	3.661	3.871	0.631	0.312	2.348	1.061
Agate	4	4.139	5.418	4.546	6.253	0.193	0.541	6.242	1.496
Biloxi	5	2.623	2.738	3.431	3.618	0.615	0.672	2.216	1.021
Agate	5	4.172	4.520	4.828	5.508	0.437	0.722	4.848	1.380
Biloxi	6	4.430	3.494	5.747	4.585	1.048	0.875	5.138	1.986
Agate	6	3.450	3.001	4.481	4.154	0.815	0.944	4.239	1.200

this condition is not thought to be the cause of floral induction, it may be correlated with the metabolism in some manner to promote the reaction or reactions causing induction.

Discussion

The results indicate that early varieties of soybeans are unsuited to many studies of photoperiodic induction because their initiation of flower primordia is not photoperiodically limited. Although floral initiation occurs on all photoperiods, the further development of the flower primordia into flowers and fruits is delayed or completely inhibited by long photoperiods. It is therefore unsafe to depend upon macroscopically visible flower buds as an indication of the effectiveness of various treatments designed to influence floral initia-

tion. If such criteria alone are used, the effects of photoperiodic treatment upon induction and upon subsequent development cannot be separated. It is important that the absence of flower primordia on control plants be assured through microscopic examination of the growing points of an adequate number of such plants. It is also more satisfactory to use varieties such as Biloxi that do not initiate flower primordia on long photoperiods until they are many months old.

In earlier work with the Biloxi soybean (1) it has been demonstrated that single dark periods varying in length from 2 to 10 days are ineffective in bringing about photoperiodic induction. Not only must there be short light periods but the intensity of these must be above a minimum level of approximately 100 foot-candles if induction is to occur. These responses indicate that light periods have an important relation to photoperiodic reaction. It has also been found that variations in temperature of the dark periods during induction produce marked differences in the effectiveness of the induction treatment. From this it seems that important photoperiodic reactions may also occur during the dark periods.

The results of the present studies show that in many varieties of soybeans darkness is not a necessary condition for floral initiation. It has been shown, however, that in a variety such as Agate, in which floral initiation is least limited by continuous light, initiation occurs much more promptly if the plants are subjected to periods of darkness. The Biloxi variety, which during its early weeks of growth does not initiate flower buds unless it receives daily dark periods of approximately 10 hours or more, finally reaches a condition after many weeks in which it is capable of forming flower primordia on considerably shorter dark periods. It is not improbable that flower primordia might be formed on the old plants of this variety grown on continuous light. The two varieties do not appear to differ in the fundamental character of the reactions that control floral initiation. They differ markedly, however, in the relative ages at which they make a similar response to long photoperiod. During the first 6 weeks, over which period chemical data are available,

they also differ in chemical composition. Agate reached a maximum in total carbohydrates at 3 weeks while the trend of the data for Biloxi seemed to indicate that a maximum had not yet been reached at the close of the experiment.

Summary

1. A study of floral differentiation has been made upon thirteen varieties of soybeans grown on nine different photoperiods, including continuous light. The varieties Agate, Mandell, Hudson Manchu, Minsoy, Mc Crostie's Mandarin, Wisconsin Early Black, Batorawka, and Mandarin initiated flower primordia on all photoperiodic treatments employed. These varieties normally mature seed in 105 days or less under field conditions. No plants of the varieties Biloxi, Avoyelles, Otootan, and Peking, and few of Tokyo, initiated flower primordia on photoperiods longer than 16 hours. These varieties require 125–175 days to mature seed in the field.

2. The initiation of flower primordia in many varieties under continuous light shows that darkness is not a necessary condition for floral initiation in them.

3. In Agate, one of the varieties that initiates flower buds most readily on continuous light, the reactions causing induction are accelerated if the plants are subjected to daily periods of darkness. Although these reactions take place in continuous light they proceed more rapidly if there is a daily alternation of light and dark.

4. Chemical analyses of the varieties Biloxi and Agate have been made at the ages of 2, 3, 4, 5, and 6 weeks. In general Agate contained less total and less soluble forms of nitrogen and more carbohydrates than Biloxi. The starch content of Agate reached a maximum at 3 weeks and declined progressively with age, while in Biloxi the trend of the data indicated that a maximum had not been reached during the experiment. The maximum carbohydrate content in Agate occurred at the time floral initiation was beginning.

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EFFECTS OF TEMPERATURE, CALCIUM AND ARSENOUS ACID ON SEEDLINGS OF POA PRATENSIS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 506

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(WITH THREE FIGURES)

Introduction

The natural range of Kentucky bluegrass over the northern half of the United States and southern Canada indicates that it is adapted to cool conditions. In addition, it has been observed (12) that bluegrass grows slowly in hot weather and, when well established, most vigorously (6) at temperatures ranging between 15° and 25° C. Also it is known that bluegrass will flourish and grow well on calcareous soils, either because of their lime content or because of other associated nutritive conditions.

During recent years there have been reports that certain chemicals, other than those usually considered as nutrients, will stimulate growth. Thus arsenic salts have been found in some cases to effect a distinct increase in growth of well established bluegrass. These observations were made, as has been most of the work, on mature plants.

Some preliminary work, however, has indicated that Kentucky bluegrass, once it has become established, will grow well under conditions which are unfavorable for the germination of its seeds and for early growth. In the experiments reported here attention is directed primarily to the effect of temperature, calcium content of soil, and arsenic on germination and on growth of seedlings.

Material and methods

Kentucky bluegrass seeds of high purity were planted in washed white quartz sand contained in 2-gallon glazed crocks. Three gm. of seeds were used in each crock, except in the final calcium carbonate series in which 2 gm. were used. The seeds were evenly distributed,

then lightly covered with sand and watered daily with distilled water until germination began. Each pot then received bi-weekly applications of a modified Shive's solution (**22**) to which had been added 0.5 ppm $ZnCl_2$, H_3BO_3, and $MnCl_2$, 0.125 ppm of CuCl, and traces of ferric citrate.

While making trial runs it was found that fine quartz sand was unsuitable for winter work, perhaps because damping-off fungi thrived under the prevailing warm, moist, cloudy conditions. The most successful practice was to use coarse quartz sand and to spray the seeds at the time of planting and at frequent intervals thereafter with a dilute Semesan solution.

At the termination of the experiments dealing with calcium carbonate fresh and dry weights of the grass were taken. Ninety days after the experiment was started half the pots in each series were sampled to determine the relative number of plants which survived; plants from 1 cm. squares along two diameters of each pot were counted.

Investigation

A. Experiments with calcium carbonate

A series of preliminary experiments were carried out in an attempt to study the effect of calcium carbonate on germination, but the results were so varied that the details are not given here.

In the final lot of the calcium carbonate series, started March 20, 1938, thirty-three pots were filled with sand which had approximately $\frac{1}{2}$ pound of calcium carbonate per pot mixed with it. The solution which dripped from the pots after watering had a reaction of pH 8.0. Although the calcium carbonate-sand mixture seemingly produced a well buffered medium, the pots were watered at intervals with water heavily charged with calcium carbonate to avoid any chance of a decrease in pH during the course of the experiment. Twenty-eight pots were set up comparably except that no calcium carbonate was added. The reaction of the leachate of the latter pots, as shown by colorimetric tests, was fairly constant at pH 5.6.

After studies were made concerning the effect of high calcium carbonate content of the sand on germination and early growth of grass; that is, some 35 days after germination, the plants of twenty-

one plus-$CaCO_3$ and of eight minus-$CaCO_3$ pots were harvested. During these early stages of growth the temperature of the sand had not been controlled, but the remaining pots were placed in tanks which kept soil temperatures constant to within 1° C. Half were maintained at a soil temperature of 15° C.; the other half at 25° C. Fifty-five days after making the temperature adjustments photographs were taken and harvests made.

B. Experiments with arsenous acid

During the months of January to March a series of experiments designed to show the effects of arsenous acid on germination and on seedling growth were set up in a greenhouse maintained at a temperature of approximately 15° C. In the first experiment five pots constituted a unit; each unit was sprayed with a liter of one of the following solutions: 0.01, 0.025, 0.05, 0.1 per cent, distilled water. The seeds were then sprinkled lightly with fine sand and moistened with distilled water.

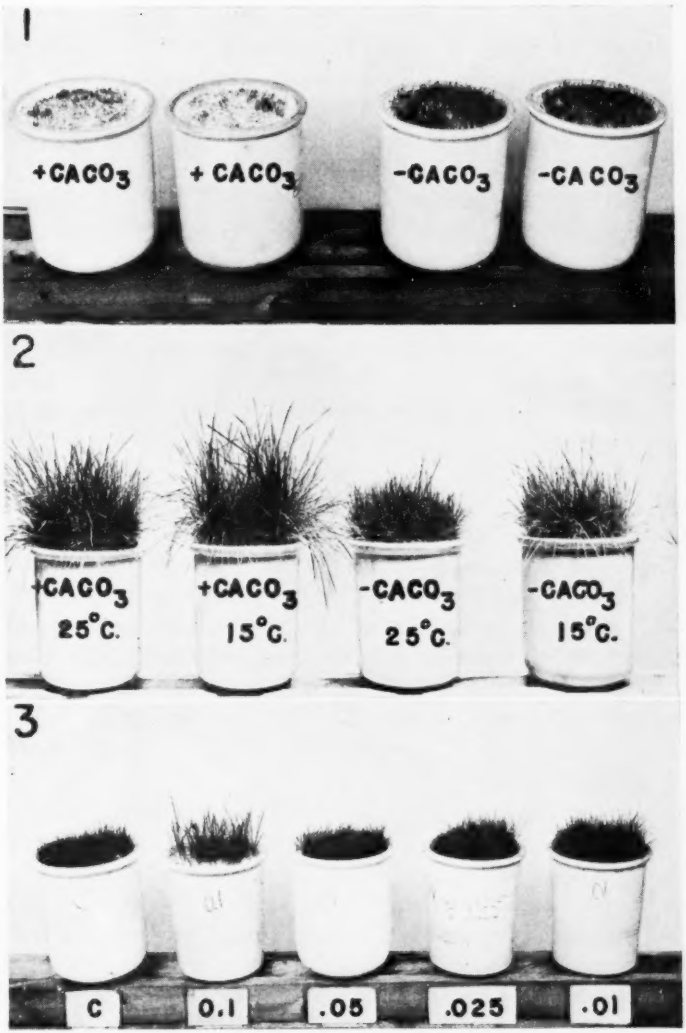
In a second experiment two pots composed a unit. The grass was grown until $1\frac{1}{2}$ months old and the same treatment given. In a third experiment the grass was $3\frac{1}{2}$ months old before treatment. All three experiments were continued for 3 months following the initial treatment.

Results

A. Effect of calcium carbonate on germination

In the initial trials of this experiment it was very difficult to get an even stand with the various treatments. In the final one, germination began 6 days after planting (March 26) in the pots lacking calcium carbonate. There were no signs of germination in the other pots. This same condition had been observed, in a less pronounced form, in a previous experiment.

Germination was delayed approximately a week in the pots containing calcium carbonate (fig. 1). After germinating, the grass would grow for only a few days and then some would wither, leaving isolated clumps. If moist chambers were placed over individual pots further germination ensued and fairly good growth followed. Even with this treatment, however, some grass died. It is not likely that



FIGS. 1–3.—Fig. 1, effect of calcium carbonate content of soil on germination of bluegrass seedlings (13 days after planting). Fig. 2, effect of calcium carbonate content of soil and temperature on growth (90 days after planting). Fig. 3, effect of arsenous acid on germination and early growth. First pot on left received no acid; pots from 0.1 to 0.01 received the indicated concentrations at the rate of 1 liter per five pots.

death can be attributed to damping-off fungi because that disease produces different symptoms.

The retardation in germination of seeds observed in the plus calcium carbonate series is in agreement with the work of RUDOLFS (20) and BORTHWICK (3). The former found that calcium nitrate had a detrimental effect upon germination and root length of a number of plants; the latter, using single and two-salt solutions, found that germination of *Hypericum perforatum* was decreased almost proportionally as the amount of calcium was increased. BORTHWICK's data indicated that pH did not affect germination to any extent but that calcium did, perhaps by changing in some way the permeability of the seed coat to water.

Shortly after germination in the calcium carbonate series the leaves appeared to be vigorous but the root systems of the seedlings were poorly developed. This may be contrasted with the grass grown without calcium carbonate, which germinated in about 6 days, produced leaves, and developed root systems rapidly.

Slow root development of the germinating seedlings in the calcium carbonate series may possibly have been caused by the low availability of phosphorus at the high pH. Phosphorus is considered necessary for development of roots, and some work with wheat seedlings (7) has indicated that its uptake is greatest in the early stages of development. It is possible that in this experiment it was a limiting factor in the early stages. Later, absorption of phosphorus was sufficient for more extensive growth.

Thirty-five days after planting, twenty-one pots treated with calcium carbonate and eight not so treated were harvested. Average lengths of tops and roots and dry weights were taken.

From table 1 it is seen that, on the basis of weight of both tops and roots, the stand of grass grown in pots at pH 8.0 was not nearly so great as that grown at pH 5.6. Nevertheless, average length of roots in the series high in calcium carbonate was almost twice as great as in the minus calcium carbonate series. Also the tops were about 1.5 cm. longer in the former series than in the latter.

The root systems of the seedlings grown at pH 8.0 were characteristically long with few or no secondary roots. Where secondary roots were present, they were short except near the growing point of

the primary root. In contrast with this, seedlings grown at pH 5.6 had shorter primary roots and a better development of secondary roots in the more mature region. Those roots grown in the presence of calcium carbonate had become fibrous in the upper portion. Most of the root was thick and white, however, indicating that it was young. The roots grown in the absence of calcium carbonate were thin and suberized almost to the tip.

Once growth had started in the pots containing calcium carbonate the plants grew more rapidly, and as uniformly (as to tops and roots) as those not having calcium carbonate. CLEVENGER (4) found

TABLE 1

EFFECT OF PRESENCE OF $CaCO_3$ ON GROWTH OF BLUEGRASS SEEDLINGS 35 DAYS AFTER PLANTING

TREATMENT	NO. POTS	PORTION OF PLANT	AVERAGE LENGTH (CM.)	DRY WEIGHT (GM.)	AVERAGE DRY WEIGHT PER POT (GM.)	TOP/ROOT RATIO
High $CaCO_3$ (pH 8.0)	21	Tops	6.6	24.2	1.15	0.52
		Roots	8.38	46.5	2.21	
No $CaCO_3$ (pH 5.6)	8	Tops	5.02	16.8	2.1	0.347
		Roots	4.8	48.4	6.05	

with oats and legumes that liming was correlated with more vigorous development. Calcium carbonate, perhaps by stimulating the natural metabolic and growth processes, is associated with a rise in the production of acids; this of course would decrease the internal pH. Such a decrease was found by BONNER in his work on *Avena* coleoptile (2) to be associated with an increase in growth substances. If this is true, more growth might be expected when calcium carbonate is present.

B. COMBINED EFFECTS OF TEMPERATURE AND CALCIUM CARBONATE CONTENT OF SUBSTRATE ON GROWTH

After the harvest, soil temperature experiments were started using the remaining pots. At the end of 55 days of growth photographs were taken and harvests made.

In general, top growth was greatest when the soil temperature

was 15° C., and the plants tended to be longer and to have more succulent blades and darker green leaves than grass grown at 25° C. (fig. 2). However, percentages of water in the leaves of the plants of the two temperature series show very little difference. This is at variance with the work of DARROW (**6**), who found in mature bluegrass that low temperature plants contained the most water per unit weight.

The grass grown in sand having a high calcium carbonate content had longer and darker green leaves than that grown on a substrate lacking lime. Furthermore, the dry matter produced was almost 33 per cent greater (table 2). The calcium carbonate series produced (although there were only about one-third the number of plants per pot) a total dry weight of tops approximately one-third greater than did those pots lacking calcium carbonate. Thus it becomes apparent that individual seedlings increase many more times in dry weight when calcium carbonate is present than when absent (table 3).

When the effects of temperature and calcium carbonate are considered, it is found that at 15° C. the presence of calcium carbonate is associated with an increase of 77.3 per cent in dry weight, and at 25° C. with 135.6 per cent, calculations being based on the dry weight of 100 seedlings.

The densest, most finely branched, and longest root systems were produced in the substrate having a high calcium carbonate content and grown at 15° C. On the basis of dry weight (table 2) the second best root system was produced in the absence of calcium carbonate and at a temperature of 15° C. These latter roots made up a fine meshwork, but they were not so near the surface as those just described; however, they were approximately as long. The roots of seedlings grown in the presence of calcium carbonate at 25° C. were somewhat similar to those grown without lime at 15° C. In general the roots were more slender, not so long, and the surface mat formation only fair. Least root growth was made by grass grown at 25° C. and lacking calcium carbonate in the substrate. Lateral growth just beneath the surface was very slight, and the roots had not penetrated to the bottom of the pots.

Table 2 indicates that low temperature is of much more importance in resulting development of abundant root growth than is

the calcium carbonate content of the substrate. In the temperature experiment it was found that root weight was 76.6 per cent greater at 15° than at 25° C. when calcium carbonate was present. Similarly growth was 91 per cent greater at 15° than at 25° C. when calcium carbonate was absent. It was found, however, that root growth was about 26.85 per cent greater when calcium carbonate was present in the soil at 15° C. than when it was not; and that it was 31.7 per cent greater when calcium carbonate was present in the soil at 25° C. than when it was absent.

TABLE 2

RELATIVE EFFECTS OF $CaCO_3$ AND TEMPERATURE ON DEVELOPMENT OF KENTUCKY BLUEGRASS SEEDLINGS

Soil treatment	Soil temperature (°C.)	No. pots	Weight roots (gm.)		Average dry weight roots per pot (gm.)	Weight tops (gm.)		Average dry weight tops per pot (gm.)	Top/root ratio*
			Green	Dry		Green	Dry		
High $CaCO_3$ (pH 8.0)	15	5	168.6	92.2	18.44	215.2	76.6	15.32	0.83
	25	5	114.0	51.6	10.32	206.5	76.3	15.26	1.48
No $CaCO_3$ (pH 5.6)	15	9	243.7	121.3	13.48	300.7	108.1	12.01	0.89
	25	9	136.5	63.5	7.05	256.9	97.7	10.85	1.53

* Calculated on basis of average dry weights per pot.

In the experiment considered here, plants having much greater green and dry weights, the greenest color, longest leaves, and a low top/root ratio were obtained in cultures grown at 15° C. Dry weights obtained by DARROW indicated that the optimum temperature for growth of mature bluegrass ranges between 15° and 25° C., while HARRISON'S (13) data from clippings indicated that the optimum temperature for top growth was 15° C.

The top/root ratios (table 2) were predominantly influenced by temperature. Those plants grown at 25° had much poorer root development than those grown at 15° C. This difference cannot be attributed to a difference in degree of competition, because there were relatively equal numbers of plants per pot in the temperature series. On the basis of these figures, calcium carbonate content of the substrate played a negligible role in altering top/root ratios. On the

other hand, dry weight figures are correlated with a marked increase in general metabolism when calcium carbonate is present.

Rhizome development, which was just beginning at the time of the harvest, was most noticeable in the pots with open and sparse growth.

The general conclusions from this experiment are that calcium in large quantities is detrimental to the germination and growth of young seedlings. Thus it would appear that heavy liming with calcium compounds before seeding would be inadvisable. Once seedlings are established, however, the application of lime would tend to stimulate growth greatly. With application of calcium carbonate there is an increase in fine roots near the surface, tending to limit the coming in of species which would have to compete for water and nutrients. In soil containing calcium carbonate, roots grow longer, which makes the grass less likely to die as a result of the drying out of upper soil layers.

TABLE 3

RELATIVE NUMBERS AND WEIGHTS OF SEEDLINGS AS CORRELATED WITH TEMPERATURE

SOIL TREATMENT	SOIL TEMPERATURE (° C.)	NO. OF 1 CM.^{2} SAMPLES TAKEN*	NO. PLANTS PER SQ. CM.	APPROXIMATE NO. SEEDLINGS PER POT	DRY WEIGHT OF 100 SEEDLINGS (GM.)†
High $CaCO_3$ (pH 8.0).....	15	56	5.4	1696.5	9.4
	25	64	5.5	1721.6	7.4
No $CaCO_3$ (pH 5.6).....	15	58	13.6	4272.58	5.3
	25	63	16.3	5120.81	3.14

* Samples taken at random along diameters of pots at right angles to one another.

† Calculated.

Planting of seeds during periods of decreasing temperatures, as in the early autumn, should prove most favorable for promoting establishment, for these experiments show that superior root development is attained at low temperatures. Since an extensive root system is necessary for survival through dry periods of the summer, roots would have a better opportunity for such development if seeds were sown in the autumn rather than the spring.

C. Experiments with arsenous acid

Sixteen days after planting and spraying the seeds there was sporadic germination of those sprayed with distilled water, and some germination of those sprayed with the lowest concentration (0.01 per cent). Two days later germination began in pots sprayed with 0.025 per cent arsenous acid. Thereafter, germination occurred in decreasing amount in the pots treated with 0.05 and 0.1 per cent acid. By the end of 27 days it was apparent that the grass treated with 0.01 per cent arsenic was germinating much more completely than the controls, while that treated with 0.025 per cent was germinating about as well as the controls. There was definite retardation in the pots treated with 0.05 per cent arsenous acid, and very marked toxicity to seeds was shown in pots treated with 0.1 per cent, the highest concentration (fig. 3). The stimulating effects continued to be apparent after 2 months in the case of the grass treated with the 0.025 and 0.01 per cent.

The slowness of germination of all the grass in the arsenic experiments may be partially attributed to the low temperature of the greenhouse. An essentially constant temperature of 18° C. was maintained throughout the day. Low constant temperatures are not most conducive to germination of Kentucky bluegrass, as was shown by Harrington (**11**); Vanha (**24**) found that temperature alternations between 20° and 32° C. are best for rapid germination.

In the experiments with the $1\frac{1}{2}$ and $3\frac{1}{2}$ months-old grass it was observed 5 days after spraying that there was injury from the 0.1 per cent solution, the leaves appearing somewhat dried. A week after spraying, injurious effects became apparent with the 0.025, 0.05, and 0.1 per cent solutions, the most severe being brought about by the highest concentration. In general the oldest grass was slowest to respond. Within 2 weeks it was apparent that the grass treated with 0.1 per cent arsenous acid would die, while in the remaining treatments, although there was injury, the severity decreased with decreasing concentration.

Slightly over a month after treatment with the various arsenous acid sprays, the older grass treated with 0.05 and 0.1 per cent solutions was almost completely killed. Thus an upper tolerance of *Poa*

pratensis for arsenous acid, when used as a spray, was found to be a 0.05 per cent solution. Some seeds germinated which had previously remained dormant. Practically all the seeds germinating in the pots treated with 0.1 per cent arsenous acid were weeds present in the seed sample, but no weed seedlings were observed in the pots sprayed with the lower concentrations. Apparently the higher concentrations stimulated germination of weed seeds. Another effect noted was that the grass sprayed with the lower concentrations was more drought resistant than the controls.

After a few weeks the older grass, which had not been injured severely, began to recover from the initial effects of arsenic "burning." The grass treated with 0.01 per cent arsenous acid, however, showed no adverse effects from spraying; there seemed to be a slight benefit.

The purpose of the arsenous acid experiments was to determine whether or not arsenic had a direct stimulatory effect on Kentucky bluegrass. The literature is filled with conflicting statements as to the effects of arsenic on different plants. Some investigators (**1, 8, 14, 23**) have maintained that arsenic, when present in small quantities, acts as a stimulating agent to growth, while others (**5, 17, 18, 19**) have found no stimulating effects. All agree that when arsenic is present in large quantities it is toxic. Very little work has been done to indicate whether the observed stimulations were direct or indirect. Some work by GREAVES (**10**) indicated that there was stimulation of soil bacteria in that there was a comparatively greater production of available nitrogenous compounds in the soil caused by the addition of arsenic. The stimulation to growth he observed may also have been caused by the inhibition of injurious bacteria and fungi. Also, according to GREAVES, arsenic can in some way liberate phosphorus from its insoluble compounds, thus exerting another indirect action.

It has long been known that different plants are differentially subject to arsenic poisoning. For example, legumes are relatively susceptible while rye is rather tolerant (**21**). HUTCHESON and WOLF (**15**) reported the use of large quantities of sodium arsenite in the control of hawkweed without injuring Kentucky bluegrass. Arsenic compounds have been found (**9, 16**) to be excellent as selective

eradicants of clover, chickweed, speedwell, and a few other low-growing weeds, while not injuring bluegrass. Toxicity is apparently most pronounced in shallow rooted species (9). In the experiments reported here seeds appear to be differentially affected by arsenous acid, a 0.1 per cent solution killing nearly all bluegrass seeds while seeds of weed grass, which were present as impurities, were apparently stimulated to germinate and grow.

Summary

1. Germination of seeds in the pots having a high calcium carbonate content (pH 8.0) began about a week after that in the pots having no calcium carbonate (pH 5.6).

2. Thirty-five days after planting, the secondary roots formed at the time of germination continued to live in the minus calcium carbonate series, while there were few or none when calcium carbonate was present.

3. The average length of roots was almost twice as great when calcium carbonate was present as when absent.

4. Ninety days after planting, top growth of grass grown on a substrate having a high calcium carbonate content was, on the basis of dry weight, approximately 33 per cent greater than when calcium carbonate was absent.

5. When the effects of temperature and calcium carbonate are considered, it is found that at 15° C. the presence of calcium carbonate is associated with an increase of 77.3 per cent in dry weight, and at 25° C. with 135.6 per cent, calculations being based on the dry weight of 100 seedlings.

6. Data indicate that calcium carbonate content of the soil is not of as great importance in producing extensive root growth in seedlings as is temperature. During the period of this experiment root growth was definitely greater at 15° than at 25° C.

7. The presence of calcium carbonate in the substrate was correlated with an evenly increased metabolic activity and growth regardless of temperature. This is especially reflected in the top/root ratios.

8. Under conditions of high temperature (22°–23° C.) liming of

the soil shortly after germination has taken place may be most favorable for the establishment of a good stand.

9. Time of germination was not delayed by the arsenic when applied as a 0.01 per cent solution, but with increasing concentration there was increasing retardation. With the lowest concentrations germination and growth were greater than in the control pots.

10. In Kentucky bluegrass an upper tolerance was found for arsenous acid (0.05 per cent), a single application of 0.1 per cent solution being sufficient to kill seeds and seedlings.

11. Harmful effects of arsenous acid were progressively less with decreasing concentration from 0.1 to 0.01 per cent.

12. Bluegrass treated with a 0.01 per cent arsenous acid solution showed greater germination than did untreated seeds. A thicker and taller stand was produced in a shorter time and this advance was maintained throughout the experiment.

13. Considerably higher concentrations of arsenous acid are required to kill the growing point of the stem than to kill the leaves.

14. Arsenous acid has a greater toxicity to Kentucky bluegrass seeds than to the seeds of some other grasses.

The writer wishes to express his appreciation of the helpful suggestions given by Dr. K. C. HAMNER during the course of these experiments.

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A MORPHOLOGICAL STUDY OF AMPLISIPHONIA A NEW MEMBER OF THE RHODOMELACEAE

GEORGE J. HOLLENBERG

(WITH THIRTEEN FIGURES)

A number of times during the last several years the writer has collected a striking marine red alga, for which the following diagnosis is given.

Amplisiphonia gen. nov.

Frond membranaceous, prostrate, horizontally expanded and irregularly lobed, attached to rocks by means of numerous unicellular rhizoids; growth marginal as a result of the activity of a row of apical cells; thallus unistratose at the margin and composed of radiating dichotomous rows of cells cut off from the apical cells, soon polysiphonous as a result of the cutting off of three upper and two lower pericentral cells from each central cell; tetrasporangia arising in modified lobes of the frond which soon become erect and are often much ruffled and bear the tetrasporangia in radiating rows; tetrasporangia arising from a special pericentral cell, more or less cruciately divided; three pericentral cells are cut off above and three below each central cell in fruiting portions; cystocarps and antheridia unknown.

Frons membranacea, prostrata, horizontaliter expansa et irregulariter lobata, ad saxa per rhizoidea numerosa unicellularia fixa; per cellulas apicales marginales crescens; margine thalli unistrato, ex seriebus radiatis et bifidis cellularum constructo; siphonibus quinque (tribus superioribus et duobus inferioribus), a quaque cellula centrale mox praecisis; tetrasporangiis in lobis modificatis prostrato-erectis frondis immersis, in seriebus radiatibus instructis, ex cellulis pericentralibus orientibus, subcruciatim divisis; in partibus fructiferis tribus cellulis inferioribus et tribus superioribus a quaque cellula centrale praecisis; antheridiis et cystocarpiis ignotis.

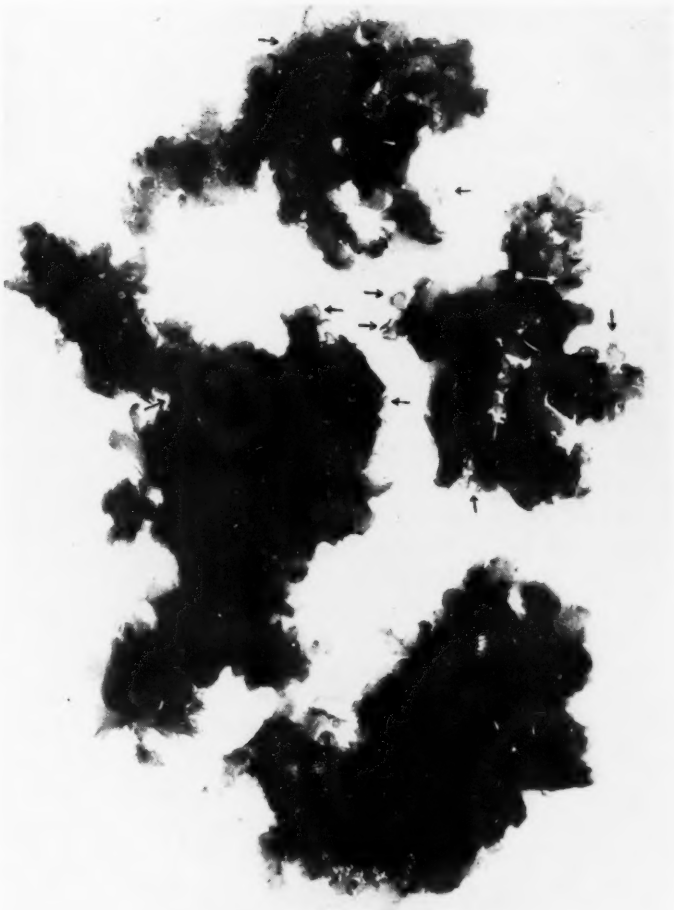


FIG. 1.—Portions of type specimen of *Amplisiphonia pacifica* gen. et sp. nov. Slightly enlarged. Arrows indicate fruiting lobes.

Amplisiphonia pacifica sp. nov.

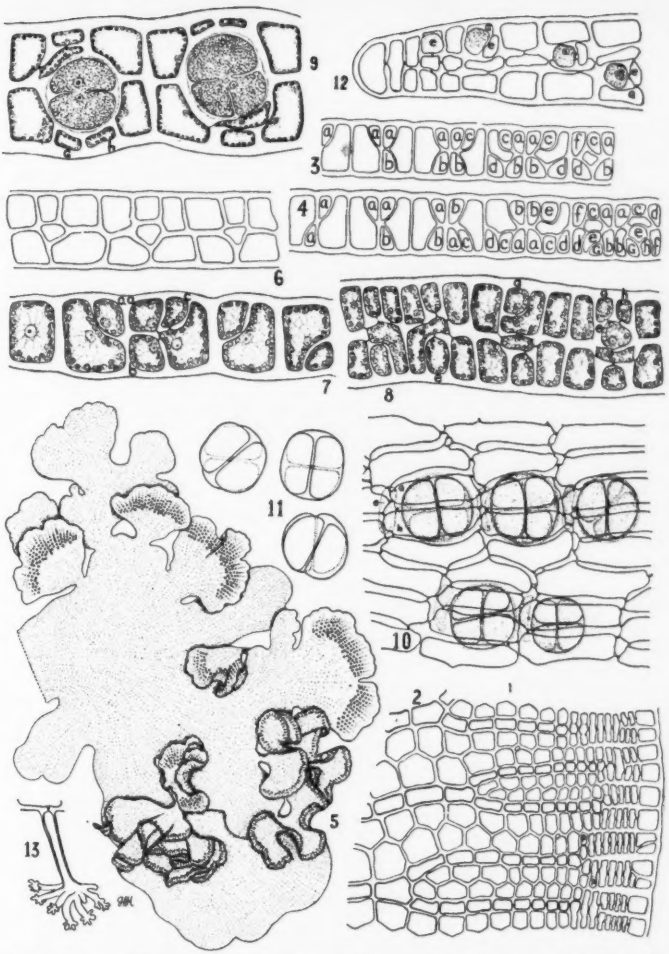
Frond prostrate, forming deeply and irregularly lobed, rosette-like expansions 10–15 cm. or up to 5 dm. across and 140 μ thick; medium or dull red in color; fruiting lobes marginal, narrowly attached, at first obovate-cuneate, prostrate and plane, later erect, ample and ruffled at the margin, up to 5 mm. high and 6 mm. or more broad.

Frons prostrata, expansa et irregulariter profuseque lobata, usque 10–15 cm. aut ad 5 dm. lata et 140 μ crassa; color nonnihil subfuscus ruber; lobis fructiferis ab margine frondis ortis, primum obovato-cuneatis et prostratis et planis, mox erectis et amplis et plicatis, ad 5 mm. altis et 6 mm. latis.

This interesting member of the Rhodomelaceae, hitherto undescribed, is frequently encountered on rocky shores along the coast of southern California. It is usually closely attached to crustaceous corallines in the lower littoral zone. It has been found clambering over the hapteres of *Eisenia*. It has been collected at all seasons of the year. It spreads in a vegetative manner and often covers a considerable area on the substrate. No. 2515, in the herbarium of the writer, has been selected as the type specimen. It is believed to represent a single plant which had spread over an irregular area 5 dm. or more in diameter. This opinion is based on the fact that, although the plants are infrequently found in fruit, in this case an abundance of fruiting lobes were present throughout the entire area covered by the plant. Only tetrasporic plants have been found so far. Most of the plants collected are sterile. Figure 1 represents portions of the type specimen collected at Corona del Mar, California, February 2, 1939. *Amplisiphonia* has been collected as far north as the Monterey region.

Methods

Both vegetative and reproductive portions of *Amplisiphonia* were fixed in a solution composed of 1 gm. chromic acid and 1 cc. of glacial acetic acid per 100 cc. of sea water. N-butyl alcohol was employed in dehydrating and imbedding, and serial paraffin sections 6 μ thick were made in the usual manner and stained with Heidenhain's iron-alum haematoxylin.



FIGS. 2–13.—All drawings made with Abbé camera lucida except sketch for fig. 5 and diagrams for figs. 3 and 4. Fig. 2, portion of margin of frond of *Amplisiphonia pacifica* showing apical cells and dichotomous cell rows and veins. Position of latter indicated by heavier lines around cells immediately above central ones; ×325. Fig. 3, diagrammatic representation of vertical section of frond near and parallel with margin, showing manner of origin of pericentral cells in vegetative portions. Fig. 4, same of fruiting lobe near and parallel with margin, showing order of origin of pericentral cells and manner of origin of supporting and cover cells. Fig. 5, part of plant of *A. pacifica* showing fruiting lobes; ×2.5. Fig. 6, transection through mature portion of frond; ×325. Fig. 7, vertical section of tetrasporic lobe of frond near and parallel with margin, showing origin of first pericentral cells; ×700. Fig. 8, same of fruiting lobe near and parallel with margin, showing final stages in formation of pericentral cells, supporting cells (*e*), and cover cells (*g*, *h*); ×700. Fig. 9, same of older fruiting portion of frond perpendicular to length of cell rows, showing tetrasporangia forming tetraspores; ×325. Fig. 10, surface view of fruiting region showing tetrasporangia, supporting and cover cells; ×162. Fig. 11, arrangement of tetraspores in tetrasporangia dissected from thallus; ×162. Fig. 12, longisection of cell row at margin of frond, showing apical cell and manner of origin of tetrasporangia; ×700. Fig. 13, mature rhizoid showing terminal branches; ×75.

Description

VEGETATIVE STRUCTURE

Growth in *Amplisiphonia* is limited to the margin of the frond and is the result of the activity of a row of relatively large marginal cells which cut off new cells by transverse divisions (fig. 2). These marginal cells measure up to 12 μ long and 9 μ wide in actively growing regions. Frequent oblique divisions of the apical cells give rise to new cell rows, and as a result the lobes of the thallus are usually round in outline. New lobes arise from the margins of old ones as a result of a local acceleration of the activity of the marginal row of cells. Radial growth of the irregularly overlapping lobes results in a rosette-like thallus in the case of older plants. The plants are perennial, and central portions of older plants are commonly composed of a number of layers of the multi-lobed thallus, each successive layer growing over and attaching to the next lower layer. The apical cells are increasingly more active toward the distal portion of each lobe, but in any limited portion of the margin the apical cells are quite uniformly active, so that the margins of the rounded lobes usually remain relatively smooth in outline (fig. 2), although they are sometimes very irregular.

At the margin the thallus is unistratose, but it soon becomes 2–3 cells thick and polysiphonous in structure. The cells resulting from the transverse divisions of the marginal cells give rise to a row of central cells from each of which five pericentral cells are cut off in vegetative portions. In older parts of the thallus the rows of central cells appear as microscopic forking veins. In figure 2 these veins are suggested by heavier lines around the cells immediately above the central cells. The first pericentral cell is cut off on the upper side of each central cell by a curving wall which severs the upper corner on the side of the cell away from its mate in the dichotomy (cell *a*, figs. 2, 3). Thus the upper outside pericentral cell is cut off first in each case. Next the lower outside pericentral cell is cut off in a similar manner. The middle upper pericentral cell is cut off next, followed usually by the inner lower cell. The upper inner pericentral cell is usually cut off last (fig. 3). No further cell divisions occur in vegetative portions except for the formation of rhizoids and of minute

"gland" cells. The latter are colorless and frequently arise in considerable number between the pericentral cells to which they are attached.

Rhizoids are unicellular and abundant, arising singly from the lower pericentral cells at the end farther from the margin of the frond. Mature rhizoids may be as much as 400 μ long and 45 μ in diameter. They are provided with lobed tips, or the tip may bear a number of short branches, each of which is terminated by a lobed expansion (fig. 13).

The cells contain great numbers of small spherical to slightly elongated chromatophores 1.5–2.3 μ in diameter and arranged in more or less tortuous beadlike rows at the periphery of the cell. Numerous secondary pit connections develop between older cells of the thallus (fig. 10).

In its vegetative structure *Amplisiphonia* is closely similar to a much smaller plant, *Placophora binderi*, first described by AGARDH (1) in 1841 as *Amansia binderi* and later (2) established by the same author as a new genus. *Placophora binderi* grows as an epiphyte on species of *Codium*. There is but one species. It is represented in the herbarium of the University of California by no. 714 of Hauck et Richter, Phykotheka Universalis. This material was collected by Madame WEBBER-VAN BOSSE along the coast of South Africa, the type locality. As stated by HOWE (4), this plant has been reported from the coast of Peru. A bit of the African material was examined by the writer and proved to be in fruiting condition. As described and figured by FALKENBERG (3), *Placophora*, like *Amplisiphonia*, develops prostrate rounded lobes which are dorsiventral in structure and attached ventrally by numerous unicellular rhizoids which arise singly. In both plants growth is marginal by means of a row of apical cells. The number and arrangement of the pericentral cells in vegetative portions are the same in both plants. In neither are there further cell divisions after the formation of the pericentral cells, except for the formation of rhizoids and of the minute gland cells common to both plants. There seems to be no previous mention of these gland cells for *Placophora*, but examination of the material from South Africa shows that they are frequently present.

FALKENBERG (3, pl. 4, fig. 7) figures a surface view of the margin

of the thallus of *Placophora*. He does not describe the origin of the pericentral cells, but his figures would indicate that they do not arise as in *Amplisiphonia*. A single row of cells is shown as forming back of each marginal cell. This seems a very improbable arrangement in view of the fact that five pericentral cells are described as forming around each central cell as in *Amplisiphonia*. Furthermore, an examination of herbarium material of *Placophora* reveals an arrangement of cell rows very similar to that herewith described for *Amplisiphonia*, three cell rows arising back of each apical cell as seen in upper surface view. It seems probable, therefore, that there is no essential difference in the manner of origin of pericentral cells in the two plants. The forking veins do not seem to show as prominently in *Placophora* as in *Amplisiphonia*, but in vegetative features *Amplisiphonia* differs from *Placophora* chiefly in the much larger size of the frond and of the cells composing it, and in its saxicolous habit. Cells of mature portions of the thallus of *Amplisiphonia* measure 140–180 × 40–50 μ; those of *Placophora binderi* are scarcely more than half as large.

Periphykon is another epiphytic member of the Rhodomelaceae with a prostrate expanded thallus of dorsiventral structure. It was described by WEBBER-VAN BOSSE (**7**) from Java. It differs from *Amplisiphonia* in several vegetative characters. Four upper and two lower primary pericentral cells are formed around each central cell. The lower remain undivided but the upper divide two or three times transversely. In *Periphykon* the rhizoids arise in bundles rather than singly as in *Amplisiphonia* and *Placophora*.

REPRODUCTIVE STRUCTURE

The chief feature which distinguishes *Amplisiphonia* from other membranous expanded members of the Rhodomelaceae with dorsiventral structure is the fact that the tetrasporangia are borne in somewhat modified, flattened lobes of the thallus rather than in cylindrical branches as in *Placophora* and *Periphykon*. These fruiting lobes are frequently very abundant but they usually arise singly at the margin of the frond. They are at first prostrate but soon become more or less erect as the outer margin expands and they become obovate-cuneate in shape. The attachment remains narrow

(fig. 5), but the margin continues to expand, and as a result the fruiting lobes become much ruffled before the outermost tetrasporangia have matured and shed their spores. Tetrasporangia mature in marked acropetal succession in an outwardly migrating zone, and the spores are shed as soon as mature. Hence in older fruiting lobes this narrow zone of mature sporangia forms a conspicuous band near the margin (figs. 1, 5). The fruiting lobes have a rounded or more or less crenate margin. They differ from the vegetative portions of the thallus chiefly in the lack of dorsiventrality, there being three pericentral cells above and three below each central cell. This lack of dorsiventrality in fruiting portions of the thallus suggests that *Amplisiphonia* evolved from some membranous but erect form without dorsiventrality similar to *Symphyocladia* or *Pollexfenia*. Vertical sections of fruiting lobes of the thallus parallel to the margin and just back of the apical cell row also show that the lack of dorsiventrality is an early and basic feature. The first pericentral cells cut off arise in a manner similar to the origin of corresponding cells in vegetative portions, but they may arise on either side of the frond (figs. 4, 7) rather than on the upper side only as in vegetative portions. Furthermore, in the case of two cell rows composing a dichotomy at the margin of the thallus, the first pericentral cell cut off from a central cell of one row may be on the same side of the thallus as the first pericentral cell cut off from a similarly placed cell in the other cell row of the dichotomy, or it may be diagonally opposite (fig. 4). Later developments occur in such a manner that the tetrasporangia appear to arise on the side of the central cell opposite that from which the first pericentral cell is cut off. It appears, moreover, that the first pericentral cell arises in a comparable position on the cells of a given cell row for a number of successive cells, and then the position of their origin may shift to the opposite side of the thallus for a similar series of cells in the cell row (fig. 12). As in vegetative portions, the second pericentral cell formed in fruiting portions is cut off directly opposite the first on the opposite side of the thallus (figs. 4, 7), and the third is cut off next to and on the same side of the thallus as the first. The fourth pericentral cell is usually cut off diagonally opposite the second, the fifth directly opposite the third, and the sixth diagonally oppo-

site the first. The order of origin may vary somewhat, the fourth and fifth cells frequently arising in reverse order.

Tetrasporangia always arise from the middle of the three pericentral cells on a given side of the thallus, and usually from the cell opposite the third (*e*, fig. 4). As already indicated, this is usually fifth in origin. Sections indicate that the third pericentral cell may occasionally give rise to tetrasporangia. The fertile pericentral cell forms the stalk cell (*e*, figs. 4, 8, 10, 12), after cutting off two cover cells (*g* and *h*, figs. 4, 8, 9, 10, 12) outwardly and a tetrasporangial cell on the side of the stalk cell toward the margin of the thallus. Hence the tetrasporangium develops between the central cell and the cover cells.

In the foregoing account it will be noted that the origin of the tetrasporangia in *Amplisiphonia* is essentially the same as that described by FALKENBERG (**3**), YAMANOUCHI (**8**), and KYLIN (**5, 6**) for *Polysiphonia* and other cylindrical members of the family, and as suggested by FALKENBERG for *Placophora*. It would seem that the shifting position of origin of tetrasporangia from one side of the thallus to the other (fig. 12), which usually occurs at a dichotomy, is to be understood as a modification of the spiral arrangement of the tetrasporangia common in *Polysiphonia* and other members of the family in which they arise in cylindrical branches. It must be kept in mind that in plants like *Amplisiphonia* and *Placophora* each row of central cells with its pericentral cells, arising as it does from a single apical cell, is to be considered homologous with a branch of *Polysiphonia*, for example. In this connection it is of interest to note that no suggestion of a spiral arrangement of the tetrasporangia was observed in the tetrasporic specimens of *Placophora* examined.

Tetrasporangia usually arise 5–7 cells back of the apical cells. Like the other cells of the thallus, they are uninucleate when first formed and contain numerous small, nearly spherical chromatophores. When the tetrasporangia are 25–30 μ in diameter, the nucleus divides to form four nuclei, after which cytoplasmic constrictions divide the protoplast into four tetraspores. Cytokinesis remains incomplete, however, until the tetrasporangia have increased to nearly their maximum size, 70–100 μ in diameter (fig. 9). The first division of the tetrasporangium is in a plane parallel with

the surface of the thallus. The half next to the cover cells then divides perpendicular to the first plane of division and also perpendicular to the length of the central cells. The inner half, next to the central cell, tends to divide in a plane perpendicular to the plane of division of the outer half of the sporangium (figs. 9, 11), but the plane of division of the inner half is frequently oblique or sometimes nearly parallel with the plane of division of the outer half (fig. 11). From the foregoing description it will be noted that the division of the tetrasporangium is approximately cruciate or quadrate, although KYLIN (6) states that in the Ceramiales division of the tetrasporangium is always in a tetrahedral manner. Moreover, the writer has observed a type of division of the tetrasporangium of certain species of *Pterosiphonia* and *Herposiphonia* very similar to that described for *Amplisiphonia*. It is perchance simply a modification of the tetrahedral manner of division associated with a dorsiventral or flattened thallus. The tetrasporangia apparently emerge in the usual manner between the cover cells. Efforts to find sexual plants of *Amplisiphonia* have so far proved fruitless.

Summary

1. *Amplisiphonia* represents a new member of the Rhodomelaceae close to *Placophora* in vegetative structure. It differs from that genus in size and habit, but chiefly in the fact that the tetrasporangia are borne in modified lobes of the thallus rather than in cylindrical branches.

2. The dorsiventral thallus grows as a result of the activity of a row of marginal cells which give rise to forking rows of cells which are laterally united and which function as central cells, cutting off three pericentral cells above and two below in regular sequence.

3. Fruiting portions of the thallus lack dorsiventrality, three pericentral cells being cut off above and three below each central cell. As in a number of other members of the family, the tetrasporangia arise from certain modified pericentral cells after the latter have cut off outwardly two cover cells in each case. They divide more or less cruciately. Sexual plants are unknown.

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STEM ANATOMY OF CHAPARRAL SHRUBS

KATHERINE S. WATKINS

(WITH FOURTEEN FIGURES)

Introduction

The chaparral formation of southern California includes numerous species of shrubs exhibiting marked similarities in habit and leaf structure (**6**). This investigation was undertaken in order to determine to what extent the dominant chaparral shrubs show a corresponding resemblance in stem structure. Collections were made over a period of a year and a half from various localities throughout southern California where typical chaparral communities occur. The following species were studied: *Quercus dumosa* Nutt., *Adenostoma fasciculatum* H. & A., *Cercocarpus betuloides* Nutt., *Prunus ilicifolia* Walp., *Rhamnus californica* Esch., *R. crocea* var. *ilicifolia* Greene, *Ceanothus spinosus* Nutt., *C. divaricatus* Nutt., *Rhus laurina* Nutt., and *R. ovata* Wats. (**13**).

Investigation

Pith

Owing to environmental conditions, growth of chaparral plants as a rule is slow (**29**). The pith is usually of small diameter, except in suckers and in shoots produced immediately following a fire. Specimens of *Adenostoma fasciculatum* obtained soon after a fire have an unusually large pith, as do also species of *Rhus* growing in less xerophytic situations. The pith of some species of *Ceanothus* is characteristically of larger diameter than that of other chaparral plants. In *Quercus dumosa* and the three members of the Rosaceae studied, lignification of the pith is uniformly heavy throughout, while in *Rhus* the lignification is uniform but slight. In *Rhamnus* and *Ceanothus* the peripheral cells of the pith differ from those of the central portion, both in their heavier lignification (**23**) and in that they remain alive while the inner cells lose their protoplasm. In many cases living cells or bands of living cells occur in the inner

portion (2). Rarely the innermost cells in *Ceanothus spinosus* escape lignification.

An abundance of stored starch occurs in the pith, especially in the smaller, more closely packed peripheral cells. Aleurone grains, crystals of calcium oxalate, tannin, or substances which are apparently glucoside-like may also occur, either associated with the starch or in separate cells. In *Rhus* small resin ducts are found in the outermost region of the pith. Small simple pits are usually abundant.

Xylem

Warming (28) states that the wood of xerophytes resembles "autumn wood"; but, with the exception of *Quercus dumosa*, which has very few vessels, all the chaparral plants studied have numerous vessels, with lumina larger than those generally ascribed to late wood. Cannon's experiments (5) show that non-irrigated desert plants have vessels more numerous and with larger lumina than those of irrigated plants. Starr (24) finds in dune plants a tendency for vessels to be more numerous in xerophytic than in mesophytic forms.

In *Adenostoma* (fig. 10), *Prunus*, *Rhamnus californica* (fig. 12), and *Rhus laurina* (fig. 14) annual rings are poorly defined, while in *Quercus* (fig. 9), *Cercocarpus* (fig. 11), *Rhamnus crocea* var. *ilicifolia*, and *Ceanothus* (fig. 13) the spring and summer wood are readily distinguishable. When present, annual rings are commonly very narrow, rarely exceeding 1 mm. in width. This condition has been shown to occur also in dune (24) and in desert plants (30). The wood is predominantly diffuse porous, tending toward ring porous in *Prunus ilicifolia*, *Rhamnus crocea* var. *ilicifolia*, and *Ceanothus spinosus*. *Quercus dumosa* shows a peculiar radially diffuse porosity (fig. 9), which is characteristic of certain other oaks as well (12). According to Webber (30), ring porous wood is characteristic of *Rhus ovata;* but I find only a very slight indication of ring porosity in this and other species of *Rhus*.

Storage tissue

The presence of abundant storage tissue in plants has been correlated with dry seasons (3). The xylem of chaparral plants con-

tains abundant storage tissue in the form of ray and diffuse parenchyma. Substitute fibers packed with starch are reported in certain desert plants (21); fibers of that nature were not observed in the chaparral plants studied by the writer. The rays are mostly uniseriate, more rarely biseriate or multiseriate. No aggregate types were found, nor were the broad rays common to many species of oaks observed in *Quercus dumosa*. The walls are always lignified, and provided with numerous pits, which in all genera studied except *Rhus* are bordered where they are in contact with the vessels. In *Quercus* both simple and bordered pits are found.

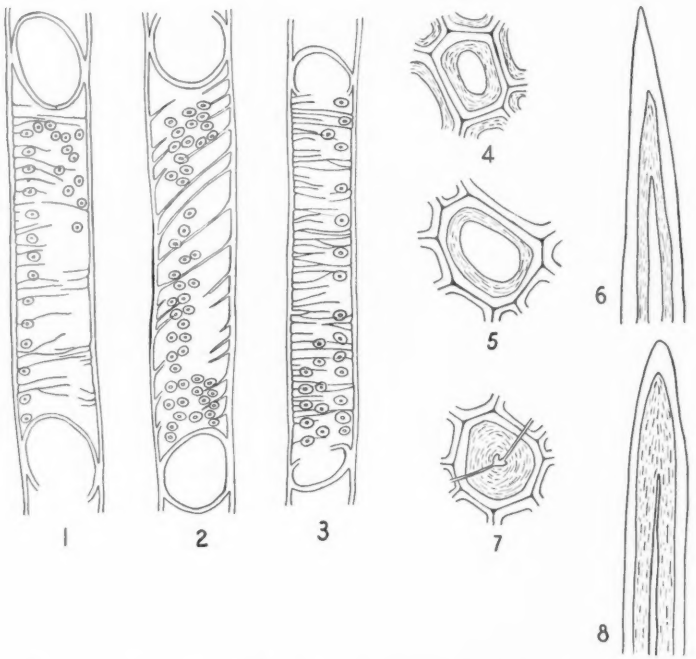
Starch grains are numerous in most of the ray cells, in *Rhus laurina* and *R. ovata* being associated with tannin, and in the Rhamnaceae and Rosaceae with glucoside-like substances. Crystals of calcium oxalate are numerous in *Quercus* and *Ceanothus spinosus*, but are rare in *Rhamnus* and other species of *Ceanothus*. As in the pith, the common storage product is starch.

Diffuse wood parenchyma is abundant in *Quercus dumosa*, *Adenostoma fasciculatum*, and *Cercocarpus betuloides*, and rare in *Prunus ilicifolia* and *Rhamnus*. In *Rhamnus* the diffuse parenchyma tends to be somewhat vasicentric. Commonly *Ceanothus* and *Rhus* have no wood parenchyma, but a few cells may be observed accompanying vessels. Calcium oxalate crystals are found in the diffuse parenchyma of *Quercus*, but are rare in *Ceanothus spinosus*. Traces of oil were noticed in *Quercus*, *Cercocarpus*, and *Rhus*, but were not sufficiently widespread to indicate importance in nutrition. Oil as a reserve material has been shown to be present in appreciable amounts in desert plants (21), even impregnating the lignified walls of the xylem and phloem elements; but in chaparral plants, as previously pointed out, starch is the common storage substance.

Water-conducting tissues

The water-conducting tissues of chaparral plants are well developed. The lignified walls of all the plants studied have bordered pits. In *Quercus* and *Rhus* these pits are in vertical rows or more or less spirally arranged. Except in *Quercus* and *Rhus*, spiral and reticulate thickenings of the pitted vessel wall are common in all species studied (figs. 1–3). In *Rhus ovata* these spiral thickenings are rarely

present. This feature has been observed in other chaparral plants and in many desert plants (30). Perforations of the end walls are simple. SOLREDER (23) reports scalariform perforations in all the species of *Quercus* studied; I find none in *Quercus dumosa*.



FIGS. 1–8.—Figs. 1–3, vessel elements: 1, *Adenostoma fasciculatum;* 2, *Prunus ilicifolia;* 3, *Ceanothus spinosus;* × 400. Figs. 4–8, mucilaginous fibers: 4, from xylem of *Rhus laurina;* 5, from xylem of *Rhamnus californica;* 6, from xylem of *Ceanothus divaricatus;* 7, 8, from phloem of *Quercus dumosa;* × 930.

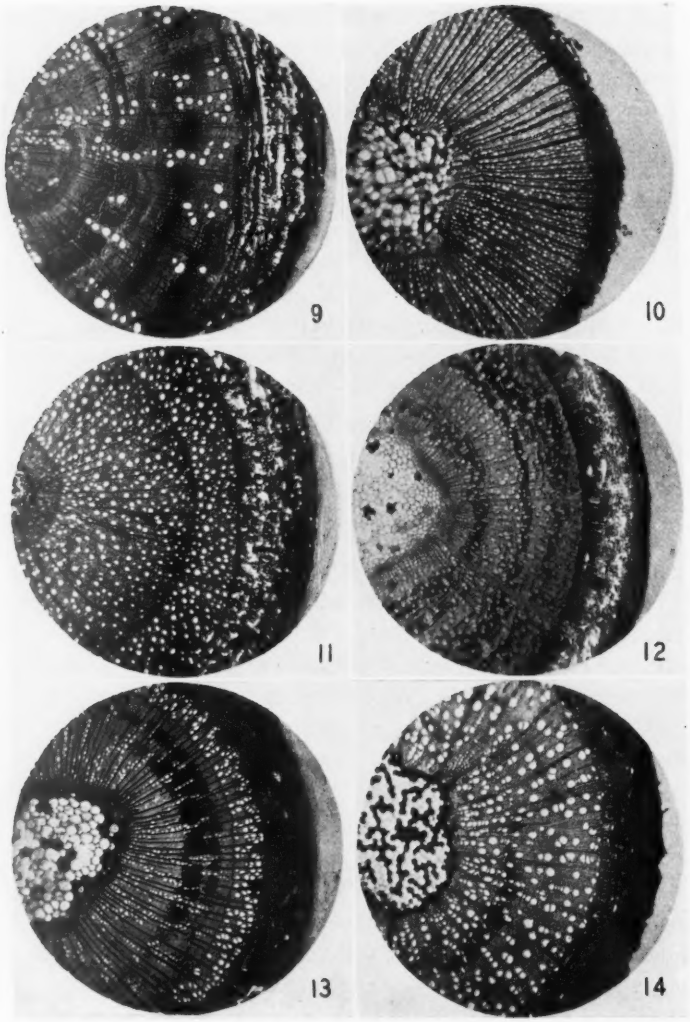
The average diameter of the lumina is variable, being largest in the vessels of *Quercus* and *Rhus* and smallest in those of *Adenostoma* and some species of *Ceanothus*. The vessel elements are comparatively short, commonly reaching a maximum length in *Quercus* of 480 μ, and a minimum length of 75 μ in *Adenostoma fasciculatum*. WEBBER (30) reports a shorter average length for a number of

desert forms. Gummosis occurs in *Quercus*, *Adenostoma*, and *Prunus*. In nearly all the plants studied a few of the vessel elements may have the lumina completely filled with gum. The tyloses commonly present in the vessels of *Quercus* were only rarely observed in *Q. dumosa*.

Tracheids are commonly absent in plants with a complex stem xylem having the functions of support and conduction definitely dissociated (**8**). In *Q. dumosa*, however, radially arranged bands of heavy-walled, border-pitted tracheids are found accompanying the vessels, apparently connecting different parts of the secondary woody cylinder. JEFFREY (**12**) reports groups of fiber tracheids lateral to the bands of tracheids in *Q. rubra;* I did not find these mechanical elements in *Q. dumosa*.

MECHANICAL TISSUES

The extensive development of mechanical tissue is generally accepted as a xerophytic character. This tissue in chaparral plants presents a number of peculiar features in addition to the heavy lignification and generally small lumina of the numerous fibers. With the exception of the three genera of the Rosaceae studied, irregularly scattered groups of fibers having only partly lignified walls were found in all cases. The distribution of these fibers is indicated in figures 9, 12, 13, and 14 by the darker stained areas. A number of investigators (**2, 8, 10, 12, 25**) report this type of fibrous element in plants of widely separated systematic affinities. The inner portion of the wall is apparently of a gelatinous consistency, and therefore is excluded from the process of lignification as outlined by ANDERSON (**1**) and others (**4, 11**). JEFFREY (**12**), EAMES and MACDANIELS (**8**), and a number of others find this type of fiber in the wood of *Quercus rubra* (**8**), in other species of oaks, in the Leguminosae (**12**), in *Morus alba*, *Betula alba*, *Diospyros virginiana*, *Eucalyptus cordata*, etc. (**2**). These investigators designate the fiber as "mucilaginous." LECLERC DU SABLON (**15, 16**) and SCHELLENBERG (**10**, p. 661) believe that this gelatinous layer is made up of hemicelluloses which are deposited late in the season and are utilized by the plant the following spring. No evidence for this use of the inner



FIGS. 9–14.—Cross sections: Fig. 9, *Quercus dumosa*, 7-year-old stem. Fig. 10, *Adenostoma fasciculatum*, 3-year-old stem. Fig. 11, *Cercocarpus betuloides*, 3-year-old stem. Fig. 12, *Rhamnus californica*, 2-year-old stem. Fig. 13, *Ceanothus divaricatus*, 2-year-old stem. Fig. 14, *Rhus laurina*, 2-year-old stem. × 20.

wall layers of these fibers, however, was found in the chaparral plants studied.

According to MANGIN (**17**), the several varieties of gums and mucilages give reactions depending on their origin from cellulose, callose, or pectic substances. A mucilage derived from a pectic or allied substance gives a bright pink-red color when treated with ruthenium red. Although this test is not specific for pectic substances, it indicates the presence of any polysaccharides containing glycuronic or galacturonic acid; for example, pectic compounds, gums, mucilages, or hemicelluloses (**14**).

In addition to giving a positive test with ruthenium red, the inner layers of the so-called mucilaginous fibers in the chaparral stems give a positive test for cellulose with chlor-zinc-iodide and other reagents (figs. 4, 5, 6). Use of the polarizing microscope offers a further check, indicating the presence of cellulose and some noncrystalline substance. This substance, as has been shown, is probably a mucilage derived from a pectic or allied compound. I have been unable to find any reference to the distribution of mucilaginous fibers in *Rhamnus*, *Ceanothus*, or *Rhus*. They have been found in a number of species of *Quercus*, but no mention of *Q. dumosa* has been made in this connection.

Typical libriform fibers also are present in the wood. These, as well as the mucilaginous elements, are provided with bordered or simple pits and are heavily lignified. No substitute fibers, such as those found by SCOTT (**22**) to form the bulk of the wood of *Parkinsonia aculeata*, were observed in the chaparral plants here studied.

PHLOEM

The cell walls of the sieve tubes, companion cells, parenchyma, and ray cells are composed of cellulose and pectic compounds. In *Quercus dumosa* the phloem ray cells become lignified, even those only one cell removed from the cambial initials. Compound crystals of calcium oxalate are nearly always found in the phloem. Solitary crystals are also present sometimes, as in *Quercus* and *Rhamnus*, forming in the parenchyma surrounding the groups of fibers to make a continuous sheath of crystallogenous cells. Chloroplasts occur in most cases, at least in the outer layers. A few starch grains are also

generally present. In *Adenostoma fasciculatum* traces of oil occur in the parenchyma. Tannin, frequently in elongated sacs, is common in the phloem of *Rhus*, and may also occur in the ray initials. The phloem parenchyma of *Quercus* and *Prunus* may contain small amounts of tannin. A characteristic feature of all the species of *Rhus* is the presence of large resin ducts in the outer region of the phloem, and of small ducts in the inner region.

The glucoside xanthorhamnin is known to be present in the parenchyma and phloem ray cells of *Rhamnus* (**7**). The writer has found similar substances in *Ceanothus* which presumably are of the same nature as the glucoside of *Rhamnus* (**19, 27, 31**). Substances which may possibly be related to l-amygdalin are found in the phloem ray cells of *Adenostoma*, and in the phloem and phloem parenchyma and rays of *Cercocarpus* and *Prunus*. *Prunus ilicifolia* may contain traces of prunasin, a glucoside reported present in *Prunus serotina*, *P. macrophylla*, and *Photinia serrulata* (**27**).

Those plants which have been referred to as having mucilaginous fibers in the xylem may have the same type of fiber in the phloem, as in *Quercus*. When present, the mucilaginous portion of the phloem fiber is more extensive than that of the xylem fiber, further reducing the size of the lumen, often almost to the point of complete disappearance (figs. 7, 8). The phloem of *Quercus dumosa*, *Cercocarpus betuloides*, and *Rhamnus* is marked by numerous groups of fibers. In *Cercocarpus* these form an almost continuous layer in the youngest region; in *Quercus* they are found in definite tangential bands accompanied by scattered stone cells; in *Rhamnus* they are surrounded by parenchyma containing crystals of calcium oxalate. *Prunus ilicifolia* and *Ceanothus* have only a few lignified fibers, occurring principally in the older region. *Adenostoma fasciculatum*, *Rhus laurina*, and *R. ovata* commonly have no mechanical tissue in the phloem.

Pericycle

The pericycle of chaparral plants is similar to that of other woody plants. *Quercus dumosa* and *Adenostoma fasciculatum* are the only two of the shrubs studied in which the mechanical tissues of the

pericycle form a continuous cylinder, consisting of groups of fibers united by stone cells. In *Quercus* these fibers are mucilaginous and in *Adenostoma* they are lignified. In *Cercocarpus*, *Prunus*, *Rhamnus*, and *Ceanothus* isolated groups of fibers are characteristic, with stone cells only rarely present. The walls of these fibers are lignified in *Cercocarpus* and *Prunus*, mucilaginous in *Rhamnus*, and unmodified in *Ceanothus*. The parenchyma between these isolated groups contains scattered chloroplasts and starch grains in *Cercocarpus* and *Prunus*, and is filled with a yellow-brown, glucoside-like substance in *Rhamnus* and *Ceanothus*. The pericycle of *Rhus* consists of arc-shaped groups of mucilaginous fibers inclosing the large resin ducts of the outer phloem.

Cortex

Collenchyma is found in the cortex of all the plants studied, with the exception of *Ceanothus* and *Rhus*. Both collenchyma and parenchyma contain chloroplasts. Scattered stone cells are found in *Quercus*, *Cercocarpus*, and *Rhus ovata*, but sclerenchyma is rarely present in the cortical regions of the other species. The inner cortical cells of *Rhamnus crocea* var. *ilicifolia* and of *Ceanothus* are much larger and thinner walled than the cells of the outer cortex, and are filled with a yellow-brown substance. This substance is also present in other cells of the cortex, pericycle, phloem, xylem rays, and pith of *Rhamnus* and *Ceanothus*.

Throughout this discussion, this substance has been referred to as a glucoside or a glucoside-like material, rather than as a mucilage (**23**) or a resin (**31**), since its reaction with potassium hydroxide to give a red-brown or purplish brown color is that reported by Tunnmann and Rosenthaler (**27**) and others (**7, 31**) for the glucosides xanthorhamnin and "rhamnokysid" (**27**) found in several species of *Rhamnus*. While the presence of glucosides in *Ceanothus* is not indicated by any of these investigators, it is very probable that the yellow-brown substances found in the cortex and elsewhere are similar to those of *Rhamnus*. *Adenostoma fasciculatum*, *Cercocarpus betuloides*, and *Prunus ilicifolia* contain a brownish or pinkish substance in the cortex and in other parts of the stem. Here, however, the ma-

terial is less abundant and gives no color reaction with potassium hydroxide other than a slight bleaching. Apparently this substance is neither mucilaginous nor resinous, but is more nearly similar to the glucosides amygdalin, prunasin, and phlorizin described for the Rosaceae (**7, 9, 26, 31**). Further investigation is necessary to show definitely that these substances present in the stems of chaparral plants are glucosides, and also to determine whether they are actually of a mucilaginous or of a resinous character. Large amounts of pyrocatechol tannin are present in the cortical parenchyma and collenchyma of *Rhus* (**18**), and traces of it occur in the cortical parenchyma and collenchyma of *Quercus*.

Extensive cork formation is common in chaparral plants, as well as in other plants growing under dry conditions (**10, 20, 25**). In all cases except *Adenostoma*, where the phellogen arises in the phloem, the periderm is subepidermal. In a few of the stems of *Cercocarpus* and *Ceanothus spinosus*, a layer of cork cells was found inclosing one or two small groups of pericyclic fibers; but whether this condition was due to injury or to the initiation of a phellogen deeper in the stem was not clear. Pyrocatechol tannin is present in the cork and phelloderm cells of *Quercus* and *Rhus*, while glucoside-like substances appear in the periderm of the other genera.

Summary

1. A comparative study of the stem anatomy of ten species, dominant in the chaparral formation of southern California, is presented.

2. The pith is lignified and generally of small diameter, containing abundant starch grains and small amounts of other ergastic substances.

3. The wood is predominantly diffuse porous, and the annual rings, when present, are narrow, rarely exceeding 1 mm. in width.

4. Reserve food is abundant in the xylem, chiefly in the form of starch.

5. Pitted vessels are numerous, mostly with spiral or reticulate thickenings on their lateral walls.

6. With the exception of the members of the Rosaceae, the presence of mucilaginous fibers in the xylem is a common feature of all

the plants studied. In *Quercus dumosa* mucilaginous fibers are found in the phloem as well.

7. Tannin is present in the phloem and cortex of *Quercus* and *Rhus*, and glucoside-like substances in the other genera.

This investigation was carried on under the direction of Professor ARTHUR W. HAUPT, to whom, as well as to Professor FLORA M. SCOTT, the writer is indebted for helpful suggestions and criticisms.

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CHEMICAL DETERMINATION OF ETHYLENE IN THE EMANATIONS FROM APPLES AND PEARS[1]

ELMER HANSEN AND BERT E. CHRISTENSEN

Introduction

In connection with physiological investigations relating to the natural occurrence of ethylene in fruits, it has been considered necessary to develop an accurate and rapid chemical method for determining the small amounts of this gas evolved from the fruits during ripening and storage. Although ethylene has been chemically identified as a constituent of the volatiles from certain fruits (**2, 4**), the procedures used are not applicable to the quantitative estimation of this gas as produced by the small experimental lots of fruit commonly used in physiological investigations. For this purpose, a micro-method based upon the bromination procedure previously described by CHRISTENSEN *et al.* (**1**) for determining the ethylene contained in plant tissues was adopted. In using this method for the determination of the ethylene evolved from apples and pears, it was necessary to ascertain whether other unsaturated hydrocarbon gases, such as acetylene, propylene, and butylene, were present in amounts sufficient to interfere with the bromination procedure used.

Analytical procedure

COLLECTION OF ETHYLENE

In order to build up a concentration of ethylene that could be determined by the micro-reagent used (0.001–0.1 ml. per 35 ml. of gas sample), approximately 1.5–2 kg. of fruit is inclosed for a definite period of time in a desiccator of known capacity. The fruit is supported on a false bottom, permitting the use of a 15 per cent potassium hydroxide solution below to absorb the carbon dioxide evolved.

[1] Published as Technical Paper no. 306, with the approval of the Director of the Oregon Experiment Station. Contribution of the Departments of Horticulture and Chemistry. The writers are grateful to VERNON H. CHELDELIN and VIRGIL HIATT for assistance in the analytical work.

Atmospheric concentration of oxygen is maintained by the addition of pure oxygen from a constant water level siphon (3). After standing at the desired temperature for a definite time (usually 48 hours), a 35-ml. gas sample is withdrawn and collected over mercury in a Hempel burette. The desiccator is then opened, thoroughly rinsed out, recharged with KOH solution, and reattached to the oxygen supply. The temperature and the barometric pressure at time of sampling should be recorded.

In using this procedure the fruit is subjected to the influence of the ethylene produced during the period of confinement. Cognizance of this fact should be taken when interpreting the data obtained.

Determination of ethylene

The procedure and apparatus used in purifying the gas sample and in determining the ethylene contained have been fully described elsewhere (1). For convenience, the essential details of the analytical method are given here.

To the burette containing the gas sample is added 1 ml. of 2.5 per cent ammonium hydroxide solution, over which the vapors are allowed to remain for 15 minutes to absorb acetaldehyde. To remove traces of any other interfering substances, the gases are passed slowly through a small Desicchlora tube (to remove excess ammonia vapor), and then through a copper coil (2 mm. I.D. × 100 cm.) immersed in a dry ice-ether mixture contained in a Dewar flask. The purified gases are collected over mercury in a Hempel burette, where they are stored until ready for analysis.

Bromination of ethylene in the purified gas sample is carried out in a reaction flask constructed from a 50 ml. Erlenmeyer fitted with a 12/30 standard taper and a capillary stopcock. The flask is charged with 5.00 ml. of 0.0025 potassium bromate, 0.5 ml. of 6 N sulphuric acid, and then partially evacuated. The gas sample and 1 ml. of N/10 potassium bromide are then introduced without breaking the vacuum. The mixture is shaken for 15 minutes on a mechanical shaker, after which 1 ml. of N/10 potassium iodide is introduced by means of the residual vacuum. The iodine liberated is titrated with 0.0025 N sodium thiosulphate from a microburette. The amount of

potassium bromate used is determined by the difference between the blank run (using air) and the actual determination. One ml. of 0.0025 N potassium bromate is equivalent to 0.028 ml. ethylene N.P.T. Since the volumes of the aliquot and the desiccator are known, the total amount of ethylene produced can readily be calculated. A correction must be made for the volume of gas remaining in the Desicchlora tube and the copper coil. In the apparatus used this amounted to 5.3 ml.

Tests for unsaturated hydrocarbon gases other than ethylene

Although reactive vapors such as acetaldehyde were removed prior to analysis, unsaturated hydrocarbon gases such as acetylene, propylene, and butylene may have been present in amounts sufficiently great to interfere with the determination of ethylene. To ascertain whether this were possible, the volatiles produced by apples and pears were treated with specific absorbents for acetylene, propylene, butylene, and ethylene. To determine the absence or presence of these gases, quantitative determinations of the unsaturated hydrocarbons contained before and after treatment were made. No procedures were used to remove carbon monoxide, since this gas would not be brominated, even if present.

The gases were collected from approximately 2 kg. each of Ortley apples and Bartlett pears inclosed in desiccators as described. Prior to analyses, 250-ml. gas samples were withdrawn from the containers and collected in a pipette over mercury. From these samples 35-ml. aliquots were taken for the solubility tests.

For removing acetylene from the gas sample, a solution of 20 per cent mercuric cyanide in 2 N sodium hydroxide was used. According to TREADWELL and HALL (**5**), this reagent removes acetylene but not ethylene from gas mixtures. Ten ml. of the mercuric cyanide solutions was placed over mercury in a Hempel burette. Approximately 35 ml. of the gas sample was then introduced and allowed to react with the reagent for 30 minutes, with occasional raising and lowering of the leveling bulb to insure complete absorption. The gas sample was then transferred to another burette containing mercury, and the unsaturates present determined. From the results

obtained (table 1) it is apparent that the unsaturates contained in the gases from both apples and pears are not soluble in mercuric cyanide solution.

For removing propylene and butylene, a solution of 87 per cent sulphuric acid was used. According to TROPSCH and MATTOX (**6**), both these gases are readily soluble in this reagent, but ethylene is not appreciably absorbed if present in concentrations less than 20 per

TABLE 1

SOLUBILITY OF UNSATURATED GASES PRODUCED BY APPLES AND PEARS IN VARIOUS REAGENTS

REAGENT USED	ML. 0.0025 N $KBrO_3$ USED PER 100 ML. GAS SAMPLE		PERCENTAGE ABSORBED (CALCULATED AS ETHYLENE)
	BEFORE ABSORPTION	AFTER ABSORPTION	
	BARTLETT PEAR		
Mercuric cyanide	6.40	6.47	None
Sulphuric acid (87%)	6.40	6.34	0.93
Mercuric nitrate	3.88	0.33	91.67
	ORTLEY APPLE		
Mercuric cyanide	4.05	4.14	None
Sulphuric acid (87%)	4.05	4.22	None
Mercuric nitrate	5.84	0.00	100.0

cent. To the burette was added 5 ml. of this solution, then 35 ml. of the gas sample was introduced and allowed to react for 3–5 minutes. The unsaturates were then determined as formerly. From the results obtained, it is apparent that the gases are not appreciably soluble in this reagent. Only 0.93 per cent of the unsaturates from the pears were absorbed, while none were removed from the apple sample.

To absorb ethylene, a solution of 20 per cent mercuric nitrate in 2 N nitric acid saturated with sodium nitrate was prepared. This reagent is used by TREADWELL and HALL (**5**) to remove ethylene

from gaseous mixtures. To the burette was added 5 ml. of this solution, then the gas sample was introduced and allowed to react for 30 minutes. The data show that the unsaturated gases from both apples and pears are highly soluble in mercuric nitrate solution. Approximately 92 per cent of the unsaturates in the gas sample from the pears was absorbed by this reagent, while that from the apples was found to be completely soluble.

It is apparent that ethylene is the active gas evolved from the apples and pears used in these experiments. If similar hydrocarbons such as propylene, butylene, and acetylene are produced, they must occur only in small amounts that would not interfere with the bromination method used for determining ethylene.

Determination of ethylene produced by apples and pears

To ascertain whether the method is applicable for the purpose intended, the amounts of ethylene produced by apples and pears at

TABLE 2

AMOUNTS OF ETHYLENE PRODUCED BY APPLES AND PEARS DURING RIPENING

Days ripened	Ml. ethylene per kg.-hour			
	Anjou pear 65° F.	Bartlett pear 42° F.	Bartlett pear 65° F.	Gravenstein apple 65° F.
2	0.003	0.113	0.222	0.100
4	0.003	0.113	0.251	0.135
6	0.000	0.101	0.055	0.220
8	0.000	.....	.....	.....
9	.....	0.135	0.000	0.280
10	0.012	.....	.....	.....
12	0.021	.....	.....	.....
14	0.030	.....	.....	.....

various stages of maturity and ripeness have been determined. The Anjou pears used were taken at time of picking, while the Bartletts had been held previously for ten weeks in cold storage. The Gravenstein apples had not been subjected to cold storage but were beginning to ripen at the start of the experiment. All samples of fruit

were held at a temperature of 65° F. during the course of the experiment, with the exception of one lot of Bartlett pears which was held at 42° F. The data are shown in table 2.

It is apparent from table 2 that the amounts of ethylene produced by both Bartlett pears and Gravenstein apples are well within the range determinable by the method described. The production of ethylene by Bartlett pears held at 42° F. remained fairly constant during the course of the experiment, while the ethylene produced by those ripened at 65° F. decreased rapidly and finally dropped below 0.001 ml. per kg.-hour when the fruit had reached an advanced stage of breakdown. The amounts produced by the Gravenstein apples increased steadily during ripening. The production of ethylene by the Anjou pears was barely within the analytical range during the early stages of ripening, but could be determined readily thereafter.

It is interesting to compare the values obtained with the data reported by GANE (2), who passed the emanations from 60 pounds of Worcester Pearmain apples for four weeks through pure bromine. By this procedure was obtained 0.85 gm. of oil, which on fractional distillation yielded 0.65 gm. boiling below 140° C. (b.p. ethylene dibromide 131.7° C.). This amount of oil as ethylene dibromide is equivalent to approximately 97 ml. of ethylene, which would be in the order of 0.005 ml. per kg.-hour. This value compares favorably with the amount of ethylene evolved by Anjou pears during the early stages of ripening but is considerably lower than that produced by Gravenstein apples.[2]

Summary

1. A chemical method based upon a bromination procedure has been adapted for determining the ethylene contained in the emanations from apples and pears.

2. Solubility tests have shown that ethylene is the active gas

[2] The following publications came to the attention of the writers after this paper had been prepared for publication.

NELSON, R. C., Physiology of ethylene production, use, and reaction in plants. Minn. Acad. Sci. **6**:37–41. 1939.

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evolved from the fruits used. Similar unsaturated hydrocarbon gases such as acetylene, propylene, and butylene were not found to be present in amounts that could be detected by the bromination procedure used.

3. The amounts of ethylene produced by several varieties of apples and pears during ripening were determined and found to be within a range of <0.001 to 0.280 ml. per kg.-hour.

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COMPARISON OF GROWTH RESPONSES INDUCED IN PLANTS BY NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID

JOHN W. MITCHELL[1] AND WM. S. STEWART[2]

(WITH TEN FIGURES)

Introduction

Some compounds capable of causing cell elongation in the pea test (7) have the following structural characteristics: (*a*) a ring system as the nucleus; (*b*) a double bond in the ring; (*c*) a side chain; (*d*) a

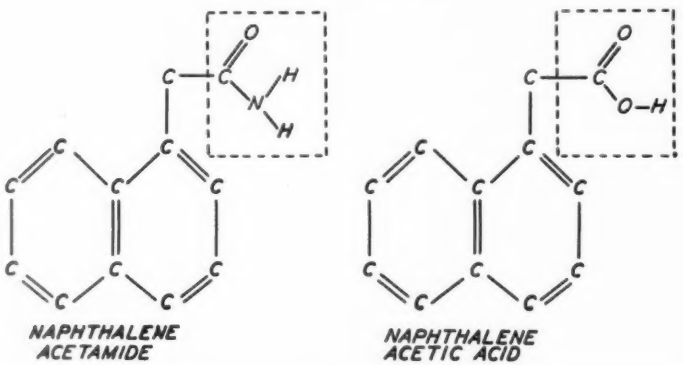

FIG. 1.—Difference in structural formulae of alpha naphthalene acetamide and alpha naphthalene acetic acid.

carboxyl group, or a structure readily converted into a carboxyl group, which is at least one carbon atom removed from the ring; and (*e*) a particular space relationship between the ring and the carboxyl group (4). Alpha naphthalene acetamide and alpha naphthalene acetic acid possess some of these characteristics in common, both being capable of inducing growth responses; but naphthalene acetamide

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does not possess a side chain with a carboxyl group. Furthermore each compound induces some specifically characteristic responses while others are common to both substances.

It has been suggested that some plants are capable of converting certain compounds (such as tryptamine, tryptophane, naphthalene aceto nitrile) that have no carboxyl side chains to their corresponding oxidized or hydrolyzed forms which contain a carboxyl group in the side chain and are then capable of inducing cell elongation (**7, 8**). Alpha naphthalene acetamide induces several responses in plants in addition to cell elongation; for example, increased secondary thickening, lignification of cell walls, root formation, delay in the formation of abscission layers (**5**), and parthenocarpy (**1**). In view of the chemical similarity of naphthalene acetamide and naphthalene acetic acid (fig. 1), experiments are reported here which were designed to compare some of the responses resulting from the application of these two naphthalene compounds to several species of plants.

Investigation

Pea test

Naphthalene acetamide and naphthalene acetic acid (**6**) were active in causing cell elongation. The former showed activity at concentrations of 100 mg. per liter. The same concentration of the latter compound was toxic, as shown by flaccidity of the tissue. This test was repeated several times with the same results. From these data it appears that naphthalene acetamide is an auxin; that is, a substance causing growth promotion by cell elongation.

Avena test

The standard *Avena* test (**8**) for growth activity of naphthalene acetic acid showed negative curvatures. These have been observed by Went and Thimann (**8**). Under the same test conditions (250 gamma per liter), naphthalene acetamide resulted in no growth curvature during the standard 90 minutes, or even after 24 hours. Negative curvatures resulted when applications were applied unilaterally at very much higher concentrations in lanolin (fig. 2). In the standard *Avena* test the concentration was possibly too low and

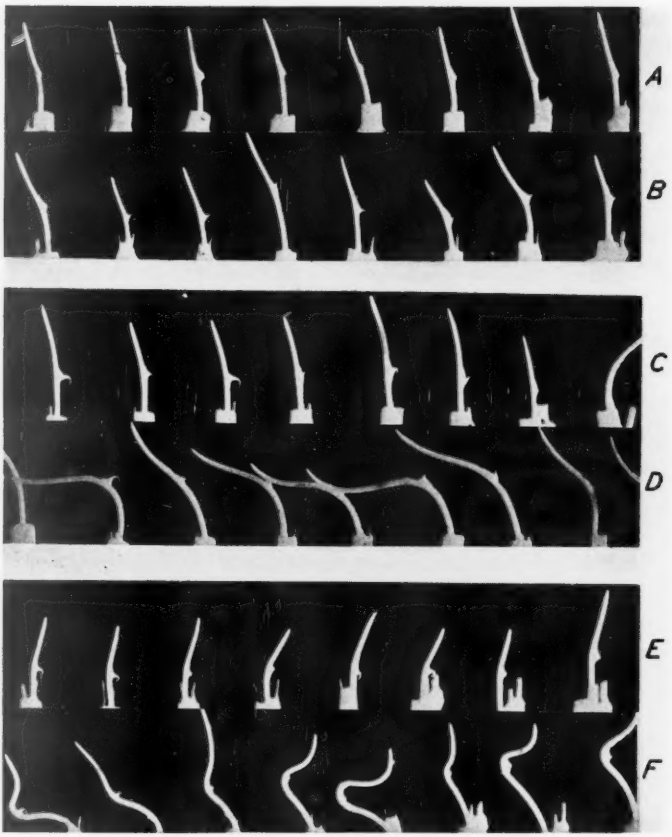

FIG. 2.—Bending responses following unilateral applications of lanolin mixtures. *A*, 0.02 per cent naphthalene acetamide after 2 hours; *B*, after 24 hours; *C*, 0.02 per cent naphthalene acetic acid after 2 hours; *D*, after 24 hours; *E*, 2 per cent naphthalene acetic acid after 2 hours; *F*, after 24 hours.

there were insufficient amounts of the acetamide or some derivative from it to act as a growth promoting substance. These experiments have been repeated three times with similar results.

Bending responses

Naphthalene acetamide and naphthalene acetic acid in lanolin applied unilaterally to rapidly growing stems caused either negative or positive bending. The response varied depending on the concen-

TABLE 1

Positive or negative curvatures* induced by unilateral applications to stems of 2 per cent mixture of lanolin and naphthalene acetic acid, naphthalene acetamide, and indoleacetic acid

Plant	Experimental conditions	Hours after application								
		Naphthalene acetic acid			Naphthalene acetamide			Indoleacetic acid		
		3	8	24	3	8	24	3	8	24
Pea	Etiolated	−	+	+	o	−	−	−	....	−
	Light grown	−	....	+	o	o	o	−	−	−
Kidney bean	Etiolated	−	+	+	o	−	−	−	−	−
	Light grown	−	−	−	o	o	−	−	−	−
Petunia	Light grown	....	....	+	....	....	−	....	....	−
Soybean	Light grown	−	−	+	o	o	o	−	−	−
Tomato	Light grown	−	+	+	o	o	o	−	−	−
Four o'clock	Light grown	....	....	+	....	....	o	....	....	−
Avena coleoptile	Etiolated	+	+	+	−	−	−	−	−	−

* Bending toward the side to which mixture was applied was designated positive.

tration of the mixture, the species, and the conditions under which the plants were grown. The results are summarized in table 1 and figures 3 and 4. Each observation was based on fifteen to thirty plants and repeated twice with the same results. With applications of 2 per cent naphthalene acetic acid to stems of etiolated kidney bean seedlings, and seedlings of tomato, garden pea, and soybean grown in the light, negative curvatures initiated during the first 3 hours were subsequently followed by positive curvatures. This effect may have been the result of an increasing concentration of naphthalene acetic acid inside the plant, as inward diffusion from the

surface continued. That low concentrations accelerated while higher concentrations inhibited cell elongation is also evident, in that a 2 per cent naphthalene acetic acid paste induced strong (19°) positive curvatures on *Avena* coleoptiles in 2 hours, while 0.02 per cent

FIG. 3.—Stem curvatures of pea seedlings 24 hours after unilateral applications of 2 per cent mixtures. Left to right: pure lanolin; naphthalene acetamide; naphthalene acetic acid (positive curvature); and indoleacetic acid (negative). Plants grown in light.

applications induced 17° negative curvatures. Two per cent applications of the same substance caused positive curvatures of tomato stems. Positive curvatures of sweet pea stems resulting from applications of 1.5 per cent lanolin mixtures of naphthalene acetic acid and negative bending from 1 per cent applications have been reported by ZIMMERMAN and WILCOXON (9).

Only negative curvatures were observed following application of 2 per cent lanolin mixtures of naphthalene acetamide. These were

FIG. 4.—Stem curvatures of etiolated kidney bean plants treated unilaterally with 2 per cent lanolin mixtures. Left to right: pure lanolin; indoleacetic acid (negative curvature); naphthalene acetamide; and naphthalene acetic acid (positive).

FIG. 5.—Response of petunia leaves to naphthalene acetamide (left) as compared with naphthalene acetic acid; 2 per cent lanolin mixtures applied to stems.

usually delayed for 12–24 hours, suggesting that possibly during this time the compound was converted slowly to a growth promoting substance, which then induced cell elongation.

In response to unilateral applications of a 2 per cent lanolin mixture of naphthalene acetamide the uppermost three or four leaf blades of petunia plants curled markedly downward (fig. 5). The stem showed a slight negative curvature distributed throughout its length. The leaf curling response did not occur with applications of naphthalene acetic acid, or indoleacetic acid. As the leaves were not flaccid the curling was apparently the result of differential growth of the upper and lower surfaces.

Emulsion sprays

To compare the effects of spray applications of naphthalene acetamide with those resulting from the use of other sprays containing naphthalene acetic acid, kidney bean plants were grown in sand with nutrient solution. The plants were selected for uniformity when 4–6 inches high and sprayed with emulsions (3) containing various amounts of the acetamide and acid respectively. Each treatment was applied to approximately 150 individual plants previously distributed at random throughout the total used.

One-half of the plants of each treatment were harvested 5 days and the remainder 10 days after treatment. They were washed free from sand and divided into roots, hypocotyls, primary leaves, first internodes, and tops (all parts above the second node). These samples were weighed, then dried in a well ventilated oven and the dry weight recorded.

Several of the responses that resulted from use of the acetamide were similar to those resulting from use of the acid. On the other hand, dissimilar responses were noted also. Thus in high concentrations (25–625 mg. per liter) both naphthalene compounds decreased internodal elongation. This response was first evident within 48 hours after treatment. Five days after treatment the heights of plants sprayed with the acid were 28, 44, and 49 per cent less than that of controls when 25, 125, and 625 mg. of acid per liter were used respectively. Leaf expansion was likewise inhibited by both compounds. Treated leaves were lighter green in color and apparently thicker

than those of controls. The margins curled evenly downward within a few days following treatment and remained permanently in this position. This effect was most pronounced in the case of the acetamide treatment.

Another response common to both naphthalene compounds was root initiation on the stems. This response was marked in the case of the most concentrated acid spray, root primordia being evident on the hypocotyl and first internode of all the plants. Roots were evident on the upper part of the hypocotyls on a few of the plants treated with the most concentrated spray of acetamide. It was also observed that the abscission of cotyledons from plants treated with both naphthalene compounds occurred several days later than from control plants.

Among dissimilar responses the most striking was the difference in the amount of bending induced by the two compounds. Within 2 hours following treatment, plants sprayed with 25, 125, and 625 mg. of naphthalene acetic acid per liter showed definite and uniform nastic response, the stems curving away from the direction of spraying. The intensity of this response varied in proportion to the concentration of spray used. Twenty-four hours after treatment plants sprayed with 25 mg. per liter had recovered and were erect, while curvatures induced with stronger concentrations were permanent throughout the remainder of the experiment. In contrast to this, the use of the acetamide resulted in no apparent curvature of stems.

Low concentrations (5 and 25 mg. per liter) of naphthalene acetamide resulted in a slight increase of growth of the tops of bean plants, as shown by dry weight measurements (table 2). Comparable concentrations of naphthalene acetic spray had no appreciable effect. Further difference in responses was observed in that 625 mg. of acetamide per liter caused approximately 48 per cent increase in the weight of roots over that of control plants, while the acid had no appreciable effect. Roots of plants sprayed with this concentration of acetamide were more fibrous than those sprayed with a comparable concentration of the acid or those of control plants.

In summary, the more concentrated sprays of acetamide stimulated root growth, inhibited top growth, and stimulated the production of relatively few root primordia from the stem. Less concen-

trated sprays resulted in increased top growth. On the other hand, the acid inhibited both root and top growth and in the concentrated sprays induced marked curvature of stems with formation of numerous root primordia.

TABLE 2

EFFECT OF EMULSION SPRAYS CONTAINING DIFFERENT CONCENTRATIONS OF NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID. FIGURES REPRESENT GRAMS DRY WEIGHT OF PARTS OF 100 BEAN PLANTS HARVESTED 10 DAYS FOLLOWING TREATMENT

Plant part	Blank	Lanolin emulsion only	Milligrams per liter			
			5	25	125	625
			Naphthalene acetamide			
Tips	65.0	64.9	68.1	75.3	53.5	24.6
Primary leaves	25.4	24.7	25.5	24.5	25.2	27.0
First internode	5.6	5.6	5.8	5.5	4.4	3.8
Hypocotyl	9.8	9.9	9.7	10.3	9.4	8.5
Roots	15.1	16.3	16.3	15.6	16.3	23.3
Total	120.9	121.4	125.4	131.2	108.8	87.2
			Naphthalene acetic acid			
Tips	46.4	59.2	46.8	44.6	40.6	0.87
Primary leaves	25.0	25.3	22.5	22.9	24.4	23.5
Hypocotyl	11.2	12.2	11.0	10.7	11.8	9.3
First internode	7.1	7.5	7.0	5.5	5.0	3.1
Roots	12.0	18.0	11.6	12.9	16.3	12.7
Total	101.7	122.2	98.9	96.6	98.1	49.5

Tumor formation, bud inhibition, and mobilization

It has been found (5) that secondary thickening, accompanied by relatively slight cellular proliferation and root initiation, was induced in stems of bean plants within a period of approximately 20 days following treatment with naphthalene acetamide. Hamner and Kraus (2) reported that treatment of bean plants with naphthalene acetic and indoleacetic acids resulted in marked cellular proliferation, tumor formation, and the initiation of numerous root primordia

within 96 hours after treatment. Experiments were conducted to compare these, and additional responses resulting from use of the acetamide, with those observed when the plants were treated with naphthalene acetic and indoleacetic acids.

Approximately 500 uniform plants were divided into four equal groups and the second internodes severed 1.5 cm. above the node to which the primary leaves were attached. Plants of each group were treated by applying approximately 2 cu. mm. of 2 per cent lanolin mixtures of naphthalene acetamide, naphthalene acetic acid, and

TABLE 3

EFFECT OF TERMINAL APPLICATIONS OF 2 PER CENT MIXTURES OF LANOLIN AND NAPHTHALENE ACETAMIDE, INDOLEACETIC ACID, AND NAPHTHALENE ACETIC ACID TO DECAPITATED BEAN STEMS. FIGURES REPRESENT GRAMS DRY WEIGHT PER 100 PLANTS

Treatment	Wet weight		Dry weight		Length of axillary shoots (cm.)
	Axillary shoots	Treated tip*	Axillary shoots	Treated tip*	
Control	307.1	11.2	28.3	1.2	4.96
Naphthalene acetamide	191.5	25.5	19.9	3.2	1.79
Indoleacetic acid	186.2	44.8	18.3	5.2	2.37
Naphthalene acetic acid	131.1	73.1	12.7	9.4	1.56

* Portion of first internode treated following decapitation.

indoleacetic acid to the freshly cut surfaces. Those of the fourth group were treated with pure lanolin as controls.

Measurements made 7 days after treatment showed that all three compounds inhibited growth of axillary shoots and induced mobilization of solid matter in the treated first internodes, as evidenced by the dry weights of these portions (table 3). Plants treated with acetamide showed no external evidence of a tumor near the region to which the chemical was applied (fig. 6), but this portion (first internode) weighed approximately 166 per cent more than comparable portions of control plants at the end of the experiment. Transverse sections made near the region treated with the acetamide showed much more secondary thickening than comparable sections from controls, the new cells arising mainly from the cambium without initiation of root primordia. In contrast to this, marked cellular

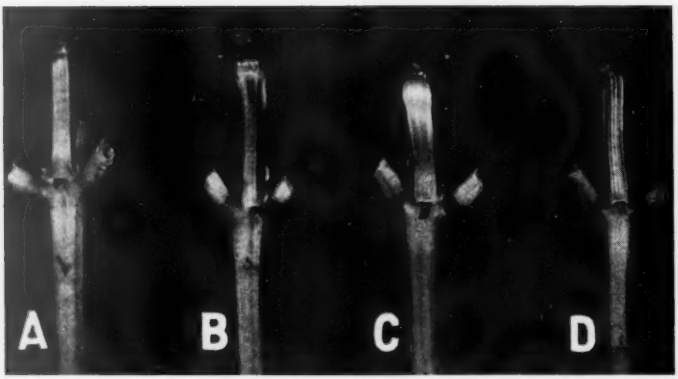

FIG. 6.—Effect of terminal applications of *A*, pure lanolin; *B*, 2 per cent mixtures of lanolin-indoleacetic acid; *C*, lanolin-naphthalene acetic acid; and *D*, lanolin-naphthalene acetamide. Five days after application naphthalene acetamide failed to induce tumor development.

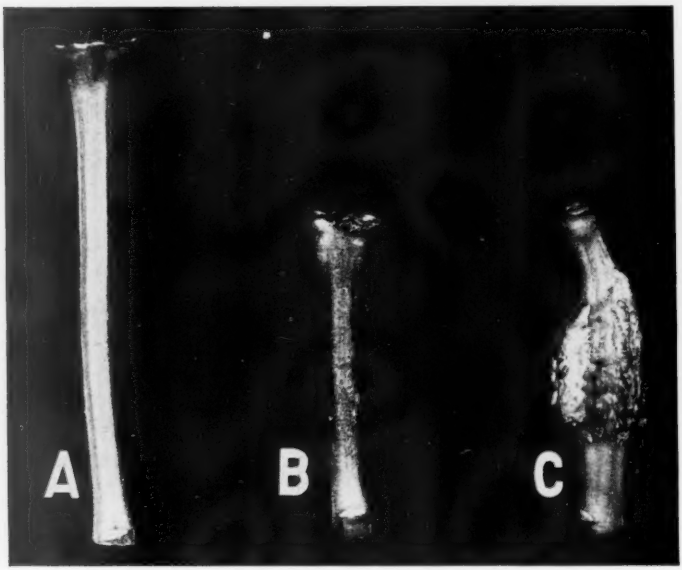

FIG. 7.—Response of first internodes of bean plants 2 weeks after treatment with: *A*, pure lanolin; *B*, 2 per cent naphthalene acetamide-lanolin paste; *C*, 2 per cent naphthalene acetic acid paste, applied as band around center of internode.

proliferation of several tissues followed application of naphthalene acetic acid and resulted in the formation of roots within 7 days fol-

FIG. 8.—Effect of ringing hypocotyl of bean cutting with 2 per cent lanolin-naphthalene acetamide mixture (left) compared with untreated cutting. Photographed 2 weeks after treatment.

lowing treatment (2). Thus mobilization of materials was associated mainly with secondary thickening in plants treated with the aceta-

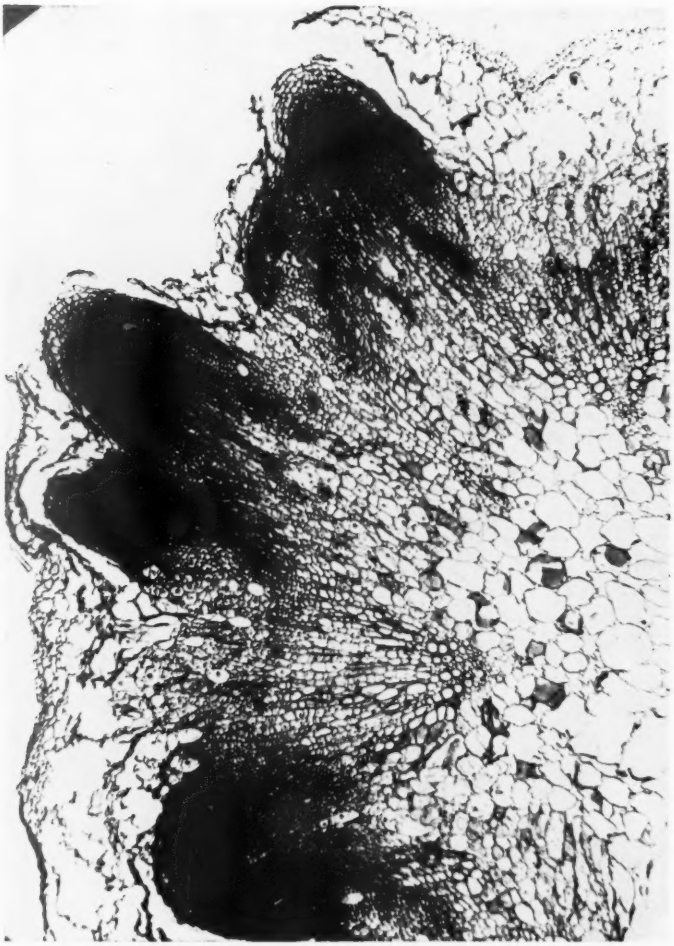

FIG. 9.—Transverse median section of tumor resulting from application (narrow ring) of 2 per cent naphthalene acetic acid-lanolin mixture 96 hours after treatment. Cells of cortex have enlarged; endodermal cells proliferated. Root primordia derived mainly from ray cells have ruptured the cortex. Pith shows little or no response. Same magnification as fig. 10.

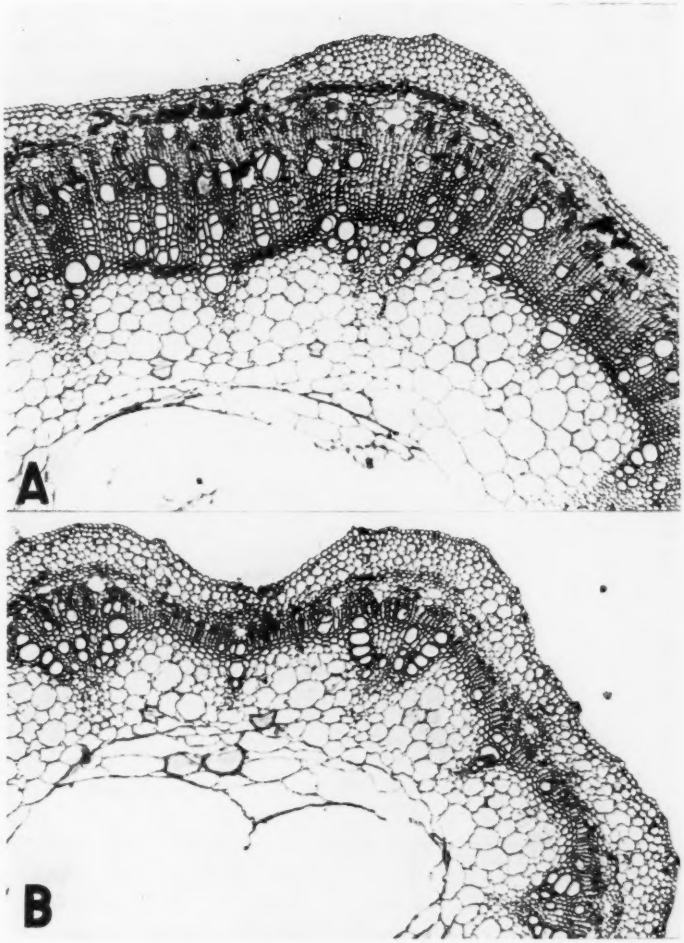

FIG. 10.—*A*, transverse median section through region over which narrow ring of 2 per cent naphthalene acetamide-lanolin mixture had been placed 216 hours after application. *B*, similar section from stem treated with lanolin after same period of time. Most obvious differences are increased thickening of walls of pericyclic fibers, somewhat wider cambial zone, much greater amount of secondary xylem, and thickening of walls of outer ray cells in *A*. *Cf.* fig. 9; same magnification.

mide (5). Naphthalene acetic acid likewise induced mobilization of materials, but in this instance mobilization was associated with marked cellular proliferation of various tissues of the stem.

Further experiments were conducted in which lanolin containing different concentrations of naphthalene acetamide and naphthalene

TABLE 4

EFFECT OF VARIOUS LANOLIN-NAPHTHALENE ACETIC ACID AND LANOLIN-NAPHTHALENE ACETAMIDE MIXTURES APPLIED AS A RING TO FIRST INTERNODES OF BEAN SEEDLINGS. FIGURES REPRESENT GRAMS DRY WEIGHT OF PARTS FROM 100 PLANTS; AVERAGE LENGTH AND DIAMETER OF 50 PARTS

Plant part	Percentage naphthalene acetic acid				Percentage naphthalene acetamide				
	2.0	0.02	0.002	0.0002	2.0	0.02	0.002	0.0002	0.0
	Dry weight (gm.)								
Leaves	53.9	58.1	58.5	57.7	66.2	58.1	58.3	58.3	61.8
Hypocotyl	20.5	19.3	20.5	17.8	17.5	16.7	17.4	17.1	15.3
First internode	20.2	12.8	11.9	11.4	8.9	11.7	10.2	9.5	9.0
Tip*	5.4	9.8	9.1	13.0	7.3	13.5	14.1	15.1	13.8
	Length (cm.)								
Petioles	3.8	5.7	6.7	6.9	4.3	6.6	6.9	6.9	7.0
Hypocotyl	6.5	6.5	6.4	6.2	6.1	6.3	6.4	6.5	6.2
First internode	3.6	4.0	4.4	5.3	3.7	4.7	5.3	5.2	5.2
Tip*	0.5	3.4	4.6	5.9	1.0	6.6	6.8	6.5	5.8
	Diameter (cm.)								
First internode†	6.4	4.9	3.7	3.6	3.4	3.4	3.4	3.2	3.4

* All above second node. † Across treated region.

acetic acid was applied as a narrow band, 2 mm. wide, around the first internodes of bean plants. The mixtures were prepared as described earlier (6) except that tertiary butyl alcohol was used as a solvent. In these experiments uniform plants grown in soil were used when approximately 10–12 inches high and having first internodes 3–4 inches in length.

Within 2 hours after treatment with the naphthalene acetic mixture, all plants except those treated with the lowest concentration showed curvatures of the stems, while none of those treated with the acetamide curved. As in the case of terminal applications following decapitation, lateral applications of the acid stimulated cellular proliferation of several tissues of the stem and initiation of root primordia (figs. 7, 9; table 4). In contrast to this response, lateral treatments with the acetamide induced more limited cellular proliferation, evidenced as secondary thickening (fig. 10). Root primordia were not initiated in the stems nor was there a significant increase in the diameter of the treated section. Both compounds inhibited development of the terminal bud (table 4).

Two per cent lanolin-naphthalene acetamide mixture greatly inhibited expansion of the terminal buds when applied in the form of a band on the hypocotyl of bean cuttings (fig. 8). Two per cent lanolin mixtures of naphthalene acetamide placed on the cut surfaces of decapitated stems of soybeans and etiolated pea seedlings likewise inhibited growth of the lateral buds.

Conclusions

The results of these experiments indicate that marked cellular proliferation was closely associated with mobilization of solid materials toward that part of the stem to which naphthalene acetic acid was applied. On the other hand, application of naphthalene acetamide also induced mobilization of solid materials, but in this case it was accompanied (at least during the interval immediately following treatment) by very little cellular proliferation and no appreciable increase in the diameter of the treated part. Although the amount of cellular proliferation induced by these two compounds was clearly different, both greatly inhibited growth of terminal and lateral buds.

The data show that most of the responses to both compounds were similar in nature, but responses following treatment with acetamide occurred more slowly than those with the acid. Responses common to both compounds were: negative curvatures in stems of etiolated pea, bean, and of *Avena* coleoptiles, and delayed tumor and root formation on beans. Dissimilar responses were also observed, those specifically associated with the acetamide being: (1) lignification of

bean stems associated with increased cambial activity, a response never obtained with application of very small amounts of naphthalene acetic acid; (2) increased and more fibrous root growth of bean plants sprayed with the acetamide; and (3) leaf curling following application of lateral treatments to stems of petunia.

Summary

1. A comparison was made of some of the responses of certain plants when treated with naphthalene acetamide and naphthalene acetic acid.

2. Naphthalene acetamide caused growth curvatures when used in the pea test, and in *Avena* coleoptiles when applied unilaterally in the form of a 0.02 per cent lanolin mixture. When applied to coleoptiles in relatively low concentrations (250 gamma per liter), using the usual *Avena* test with agar blocks, the acetamide failed to give growth curvatures. Naphthalene acetic acid caused growth curvatures in both the pea and *Avena* test at concentrations of 1 mg. per liter and 250 gamma per liter, respectively.

3. Unilateral applications of lanolin containing naphthalene acetamide (2 per cent) on stems of etiolated pea and kidney bean plants resulted in negative curvatures. No apparent curvatures resulted when the stems of four o'clock, soybean, and tomato plants grown in natural light were treated unilaterally with acetamide. Petunia and kidney bean plants treated in this manner showed slight negative curvatures. Applications of a 2 per cent mixture of lanolin and naphthalene acetic acid resulted in first negative then positive bending in all except kidney bean plants grown in light. Negative curvatures were interpreted as resulting from initially low concentrations of the acid. Growth was apparently inhibited, on the side of the stem nearest the application, as the concentration of acid in the tissues became greater.

4. Naphthalene acetamide and naphthalene acetic acid were applied to bean plants in the form of emulsion sprays. Both compounds inhibited development of the terminal buds and expansion of primary leaves when used in strong concentration. The acetamide stimulated root growth over that of untreated plants while the acid was ineffective in this respect. The less concentrated acetamide

sprays accelerated top growth to some extent while the acid sprays had no noticeable effect.

5. Terminal applications of 2 per cent lanolin-naphthalene acetamide or naphthalene acetic acid mixture to decapitated beans or lateral applications on the stems resulted in mobilization of solid materials toward the treated region. Mobilization was associated with appreciable secondary thickening in plants treated with naphthalene acetamide, and with marked cellular proliferation when naphthalene acetic acid was applied.

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GROWTH SUBSTANCES AND GAMETIC REPRODUCTION BY PHYCOMYCES[1]

WILLIAM J. ROBBINS

(WITH FIVE FIGURES)

Introduction

Although numerous observations (3) on the formation of zygotes by the Mucoraceae were made by the TULASNE brothers, DE BARY, BREFELD, VAN TIEGHEM, LE MONNIER, BAINIER, THAXTER, and others after the first observation by EHRENBERG on *Sporodinia grandis* in 1820, a correct understanding of the process dates from the studies of BLAKESLEE (1). BLAKESLEE'S discovery that *Phycomyces* is heterothallic made possible an attack on the physiology of gametic reproduction of that organism, and several investigators have made contributions to the problem.

ORBAN (5) reported that 1 per cent $MnCl_2$ added to a 3 per cent biomalt and 2 per cent agar medium permitted vigorous vegetative growth but inhibited gametic reproduction. In fact, additions of NaCl, $Ca(NO_3)_2$, and other salts decreased zygote formation, caused the development of various abnormalities, and at higher concentrations inhibited gametic reproduction.

SWARTZ (17) and RONSDORF (7) found that the most favorable temperature for zygote production was 20° C. or somewhat lower, and in their experiments at temperatures above 22° gametic reproduction was reduced or entirely inhibited.

BURGEFF (2) presented evidence that substances from the plus strain diffusible through a celloidon membrane were responsible for the initial development of zygophores of the minus strain, and vice versa. RONSDORF (7) found that the larger amounts of nitrogen inhibited formation of zygotes, in agreement with the observations by BENECKE, KLEBS, and others for other plants.

Study of the nutritive aspects of gametic reproduction by

[1] Assistance in this work was furnished by the personnel of Works Progress Administration Official Project 465-97-3-70. Special thanks are due V. N. SERGIEVSKY.

Phycomyces was handicapped, however, because growth of the organism could be secured only on media to which some supplement of natural origin was added; for example, an agar containing 3 per cent malt was frequently used. Supplements of natural origin are variable in character and complex in makeup, and their effects are therefore difficult to evaluate.

In a series of papers published from 1931 to 1934, SCHOPFER reported observations on the formation of zygotes by *Phycomyces blakesleeanus*. He observed that little growth developed and few or no zygotes were produced on an agar containing pure sugar, asparagine, and mineral salts. Additions to this medium of dried brewers' yeast, extracts of yeast, vitamin B concentrate, malt extract, extracts of rice polishings, wheat germ (**10, 13, 15**), or the substitution for the pure sugar of impure maltose or impure sucrose (**12**) caused material increase in growth and the formation of 1000 or more zygotes in a single petri dish. SCHOPFER (**8, 9**) concluded that vitamin-like substances are concerned in the growth and in the gametic reproduction of *Phycomyces*.

In 1934 SCHOPFER (**11, 14**) reported that the addition of crystalline thiamin (vitamin B_1) to an agar medium containing pure sugar, asparagine, and mineral salts permitted growth of *Phycomyces* and induced the development of zygotes. His discovery that *Phycomyces* would grow vigorously in a solution of sugar, asparagine, mineral salts, and thiamin and the availability of all these substances in chemically pure form permit further investigation of the nutritive aspects of gametic reproduction by this fungus.

Although SCHOPFER was not certain that thiamin was the sole effective agent in the preparations of natural origin which he had found beneficial for *Phycomyces*, the conclusion to be drawn from his work is that a medium of sugar, asparagine, mineral salts, and thiamin is adequate for its growth and gametic reproduction.[2] Several investigators (including the writer) as well as later work by SCHOPFER have amply confirmed his original findings on the adequacy of such a medium for the vegetative growth of *Phycomyces;*

[2] SCHOPFER (**14**) gives the following as a synthetic medium necessary for *Phycomyces:* glucose 10 per cent; asparagine 0.2 per cent; $MgSO_4 \cdot 7H_2O$ 0.05 per cent; KH_2PO_4 0.15 per cent; crystalline thiamin (vitamin B_1) 25 gamma per cent.

and at temperatures of 20° or somewhat lower gametic reproduction occurs on the same medium. At 25° C., on a medium supplemented with thiamin alone, gametic reproduction is absent or scanty, and experiments at that temperature reported in this paper suggest that growth substances in addition to thiamin are concerned in the gametic reproduction of *Phycomyces*.

Material and methods

The plus and minus strains of *Phycomyces blakesleeanus* were originally secured from Dr. A. F. BLAKESLEE. Two basic culture solutions were used, each of which was supplemented with 0.01 ppm boron, 0.01 ppm molybdenum, 0.20 ppm iron, 0.04 ppm copper, 0.15 ppm zinc, and 0.02 ppm manganese. Solution 1 contained per liter 50 gm. dextrose, 0.5 gm. asparagine, 0.5 gm. $MgSO_4 \cdot 7H_2O$, 1.5 gm. KH_2PO_4, and 0.25 mg. thiamin. Solution 2 was solution 1 with 100 gm. dextrose and 1 gm. asparagine. The asparagine was purified by recrystallization from alcohol; the thiamin was Merck's synthetic; the other chemicals were of chemically pure grade. Difco standardized agar was used. Petri dishes 10 cm. in diameter and 1.5 cm. deep were used as culture vessels and 25 ml. of medium was placed in each dish. Sterile conditions were maintained throughout; the media were sterilized in an autoclave at 15 lb. pressure for 20 minutes. Drops of a spore suspension of *Phycomyces* in sterile distilled water were used as inoculum and the cultures were incubated at 25° C., except where otherwise noted, in weak diffuse daylight. The formation of progametes and zygotes was observed under a hand lens or a binocular microscope. Only those zygotes were recorded which showed black appendages partially or completely developed; no attempt was made to distinguish separately those which were fully matured.

Experimentation

Interest in this problem had been aroused by difficulties experienced in obtaining zygotes of *Phycomyces* with media upon which they were expected to develop. It was assumed that a solution of sugar, asparagine, mineral salts, and thiamin would be satisfactory, but repeated experiments with such medium solidified with agar

gave negative or unsatisfactory results under the conditions employed.

Growth on media to which no thiamin was added was extremely scanty; few or no sporangiophores were produced and no progametes or zygotes developed. When thiamin was added growth was profuse, aerial mycelium developed, abundant sporangiophores and sporangia were produced, but few or no progametes formed. The production of progametes and zygotes was materially increased, however, if in

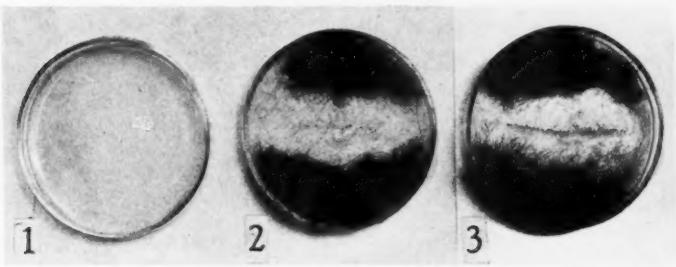

FIG. 1.—Effect of thiamin and oatmeal extract on gametic reproduction. All petri dishes contain same amounts of dextrose, asparagine, and mineral salts and 1 per cent agar. *1*, no thiamin; *2*, 0.00625 mg. thiamin; *3*, 0.00625 mg. thiamin and extract of 0.625 gm. oatmeal. Age 11 days.

addition to thiamin an extract of some material of natural origin was included with the agar, sugar, asparagine, and mineral salts.

This is illustrated in figure 1. In this instance solution 2 with 2 per cent agar was used. The medium in each dish was inoculated at opposite edges with the plus and minus strains and the cultures were photographed after 11 days of incubation. Growth on agar containing dextrose, asparagine, and mineral salts but no thiamin was too sparse to show in the photograph. On the same medium plus thiamin the growth was profuse and abundant sporangia were formed. The line of progametes was too thin to be visible in the photograph, however, and a total of seven zygotes with black appendages partly or completely developed was formed on four dishes—fewer than two per plate. The addition of a water extract from 0.625 gm. of oatmeal[3] per plate to the medium containing thiamin resulted in a

[3] The dry matter in the extract added to each petri dish was 14 mg.

heavy line of yellow progametes and increased the number of zygotes nine times.

Other experiments carried out in the same way with a 1 per cent agar containing dextrose, asparagine, mineral salts, and thiamin gave entirely negative results, so far as progametes and zygotes were concerned, when no extract was added to the medium.

A comparison of these experiments with those of SCHOPFER (**14**) revealed certain differences. In addition to the difference in strains of the fungus employed and in the source of the chemicals, SCHOPFER used 3 per cent agar instead of the 1 or 2 per cent in my media; his temperature of incubation was 18° to 20° C. and mine 25° C.; he inoculated the petri dishes in six places arranged in two parallel lines about 3 cm. apart, as judged from the photographs reproduced in his paper. Although some of these differences were originally considered insignificant, it seemed advisable to determine their importance as well as that of certain other factors.

METHOD OF INOCULATION

In the original experiments the agar medium in the petri dishes was inoculated at one edge with a drop of a spore suspension of the plus strain of *Phycomyces*, and at the opposite edge with the minus strain. The distance between the two drops was about 8.5 cm. SCHOPFER used six drops and the distance between the inocula of the plus and minus strains appears to have been about 3 cm. Furthermore, in one or two instances in my first experiments the dishes were tilted in handling and the drops of inoculum ran over the surface of the agar, bringing the mycelia close together from the beginning of the growth period. Under such circumstances progametes were formed on the same medium on which none developed when the inocula remained in their original locations near opposite edges of the dish.

Because of this observation the effect of the method of inoculation was investigated. Three drops of a spore suspension of the plus strain were equally spaced in a line across the dish, and three drops of the minus strain were arranged in a line parallel with the first and 2.5–3 cm. away, with the result that the initial mycelia of the plus and the minus strains were 2.5–3 cm. apart instead of 8.5 cm. as in

the first method of inoculation described. Because of the shorter distance separating them the two mycelia met earlier in their growth. With this method of inoculation progametes and zygotes were produced regularly on agar media on which the other method of inocula-

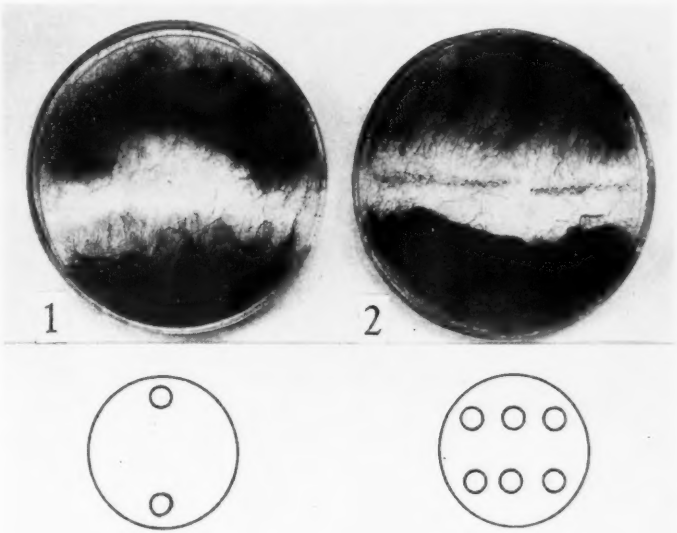

FIG. 2.—Effect of method of inoculation on gametic reproduction. Both dishes contain 1 per cent agar with same amount of dextrose, asparagine, mineral salts, and thiamin. *1*, inoculated at opposite edges of dish; *2*, inoculated in six places arranged in two lines about 3 cm. apart. Sketches show method of inoculation. Age 11 days.

tion gave little or no result (fig. 2). In this experiment the medium consisted of solution 2 containing 1 per cent agar.

It might be suggested that the distance separating the inocula was not the factor responsible for the difference in gametic reproduction noted, but that the results were related to the quantity of inoculum. In the first method only one drop each of a spore suspension of the plus and minus strains was used while in the second three drops of each were used in each plate.

That distance between the inocula and not quantity of inoculum

was the important factor was demonstrated by placing a drop of a spore suspension of each of the two strains at different distances from one another on media in petri dishes.

In one experiment solution 1 containing 1 per cent Difco agar was prepared and poured into petri dishes. Quadruplicate plates were inoculated with one drop of a spore suspension of each strain, the drops being placed 1, 2, 3, 5, 6, and 8 cm. apart. In addition, in one set of plates three drops of each strain were arranged in parallel lines

TABLE 1

DISTANCE SEPARATING INOCULA AND DEVELOPMENT OF PHYCOMYCES. ALL GROWN ON SAME 1 PER CENT AGAR MEDIUM CONTAINING DEXTROSE, ASPARAGINE, MINERAL SALTS, AND THIAMIN (FIG. 3)

DISTANCE SEPARATING INOCULA (CM.)	MYCELIA MET AFTER	PROGAMETES OBSERVED AFTER	LENGTH OF LINE OF PROGAMETES (CM.)	YOUNG SPORANGIOPHORES FORMED AFTER	NO. ZYGOTES PER PLATE
1.0	30 hours	66 hours	5.0–5.5	66 hours	9
2.0	49	66	4.0–6.0	66	15
3.0	62	98	4.5	66	2
5.0	66	100	3.0	66	1
6.0*	98	None	.....	66	0
8.0	98	None	.....	66	0
3.0†	66	68	8.5	66	4

* One culture in four showed a few progametes.

† Inoculated in six places in two parallel lines about 3 cm. apart.

about 3 cm. apart. Observations were made at intervals in order to fix as nearly as practical the time when the mycelia of opposite strains met, the time progametes were observed, and the time young sporangiophores appeared. After 10 days the length of the line of progametes was measured and the number of zygotes with appendages was counted (table 1; fig. 3).

No progametes were produced in the plates when the original inocula were 8 cm. apart and only one plate in four showed a few when the separating distance was 6 cm. When the distance between the original inocula was 1, 2, 3, or 5 cm., lines of progametes were produced; but at the latter two distances the line was short. The superiority of the shorter distances between the inocula was evident also in the number of zygotes with appendages. When the inocula

were 1–2 cm. apart, nine or fifteen zygotes per plate appeared; for greater distances the number was reduced to two, one, or none (table 1).

Why should the distance between inocula make a difference in the gametic reproduction of *Phycomyces?* An unqualified answer cannot

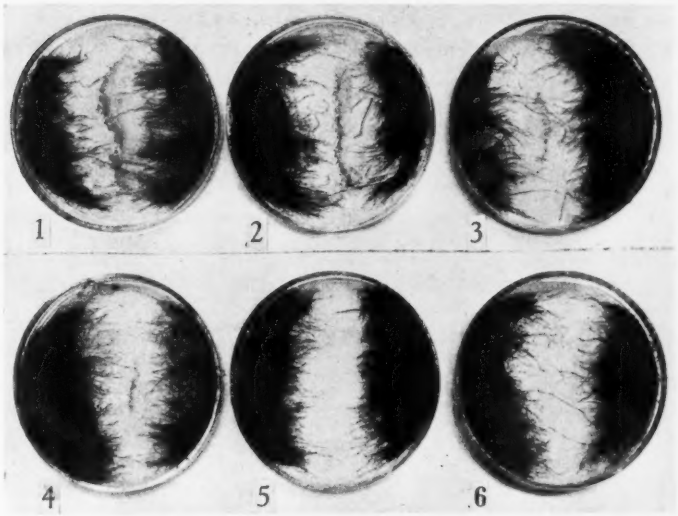

FIG. 3.—Distance between inocula and gametic reproduction. All dishes contain 1 per cent agar with same amounts of dextrose, asparagine, mineral salts, and thiamin and were inoculated with a drop of spore suspension of plus and minus strains. *1*, distance between inocula 1 cm.; *2*, distance 2 cm.; *3*, 3 cm.; *4*, 5 cm.; *5*, 6 cm.; *6*, 8 cm. Age 9 days.

be given to this question. There seemed to be some relation between the age of the mycelium and the development of the progametes. If none was formed within 3 or 4 days from the time of inoculation, none developed later. In fact, unless the opposing mycelia grew together within 3 days from the time of inoculation, no progametes were produced under the conditions of these experiments. Formation of sporangiophores and sporangia seemed to be associated in some way with inhibition of the production of progametes. If sporangiophores and sporangia developed freely before the two op-

posite mycelia grew together, formation of progametes was likely to be inhibited. It appeared, furthermore, that once production of sporangia was well under way the ripening and maturation of the zygotes stopped.

This apparent antagonism between the formation of sporangiophores and gametic reproduction in *Phycomyces* would be understandable on the assumption that growth substances other than thiamin are concerned in the development of this organism, and that these substances are exhausted by the formation of sporangiophores and sporangia, either by being used in the formation of these agametic structures or by being destroyed by autolysis (which probably begins with the production of sporangia).

Concentration of agar

Schopfer (**14**) used a 3 per cent agar in his experiments on the effect of crystalline thiamin on gametic reproduction by *Phycomyces*.

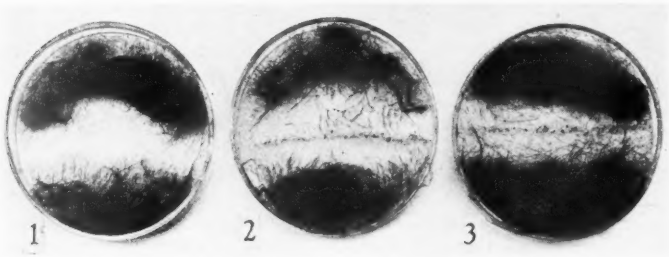

Fig. 4.—Effect of concentration of agar and of potato extract on gametic reproduction. All dishes contain same amounts of dextrose, asparagine, mineral salts, and thiamin and were inoculated at opposite edges with plus and minus *Phycomyces*. *1*, contains 1 per cent agar; *2*, 3 per cent; *3*, 1 per cent agar and per plate the extract of 11 gm. of potato. Age 11 days.

It appeared that the concentration of agar was of importance in my experiments; more profuse gametic reproduction was secured on the higher concentrations. When the distance between inocula of the two strains was 8–8.5 cm., progametes were rarely secured at 25° C. on a 1 per cent agar made up with solution 1 or 2. On a 2 per cent agar progametes were formed but few zygotes developed; with 3 per cent the development of both was more profuse.

The effect of agar concentration is shown in figure 4. In this in-

stance solution 2 was used. The dishes were inoculated with the plus and minus strains at opposite edges and photographed after 11 days. On the 1 per cent agar no progametes were formed although the vegetative growth was good. On the 3 per cent agar a line of progametes developed and zygotes were produced. It is of interest to note that the addition to the 1 per cent agar medium of the extract of 2.75 gm. of potato[4] per plate gave results similar to those found on the 3 per cent agar to which no such natural supplement

TABLE 2

EFFECT OF CONCENTRATION OF AGAR ON GAMETIC REPRODUCTION BY PHYCOMYCES

Medium	Two inoculations 8.5 cm. apart		Six inoculations in two lines 3 cm. apart	
	Progamete production	No. zygotes per plate	Progamete production	No. zygotes per plate
Solution 2 plus 1% agar....	None	None	Line 2 mm. wide	3
Solution 2 plus 3% agar....	Line 2 mm. wide	31	Line 3 mm. wide	56
Solution 2 plus 1% agar....	.....	.....	Line 2 mm. wide	4
Solution 2 plus 3% agar....	.....	.....	Line 3 mm. wide	46

was added; in other words, the higher concentration of agar without potato extract had much the same effect on gametic reproduction as the addition of potato extract to the lower concentration.

Table 2 summarizes the results of experiments in which the effect of 1 per cent and 3 per cent agar was compared. The superiority of a medium containing 3 per cent agar over the same medium containing 1 per cent is shown. The favorable effect of the higher concentration was evident both when two inoculations at opposite edges of the dish were used and when six inoculations in two lines about 3 cm. apart were employed; although in every instance superior results were obtained with the latter method. SCHOPFER (9) also found the higher concentrations of agar favorably to affect gametic reproduction by

[4] The dry matter in the extract added to each petri dish was 44 mg.

Phycomyces. He considered, however, that the agar did not exert a chemical action, but that its effect was physico-chemical because of its density, cohesion, and osmotic pressure resulting from its colloidal character. Apparently agar contains substances which beneficially affect gametic reproduction; otherwise it would seem difficult to explain the similarity of the results obtained by increasing the concentration of agar and those secured when potato extract was added to the more dilute agar. Furthermore, the beneficial material can be extracted in part from the agar.

Effect of purifying agar

If the assumption is correct that agar contains some substance or substances important in gametic reproduction, then it should be possible to remove these substances and reduce the effectiveness of the agar. An attempt to purify agar by extraction with methyl alcohol, or by aqueous pyridine, was partially successful.

Difco agar was dissolved in water by heating and the hot liquid poured with stirring into redistilled methyl alcohol. The precipitate was centrifuged off, redissolved in hot water and reprecipitated with alcohol, removed by centrifuging, washed with 60 per cent methyl alcohol, and dried. The methyl alcohol extracts were combined and evaporated by boiling to small volume. About half the agar remained in the extract.

Solution 2 was prepared with 1 per cent Difco agar, 1 per cent Difco agar purified with methyl alcohol, and with the methyl alcohol extract sufficient to make a 1 per cent agar.

The plates (in quadruplicate) were inoculated in six places with the two strains of *Phycomyces*. On the Difco agar a line of progametes was produced and six zygotes per plate developed (table 3). On the purified agar the line of progametes was thinner and the zygotes fewer. The line of progametes on the medium prepared with the extract was intermediate in thickness between these two and the number of zygotes was no greater than on the purified agar. This indicated that there were materials in Difco agar beneficial to gametic reproduction and that they could be extracted in part with methyl alcohol.

Dry powdered Difco agar was extracted at room temperature over

a period of 48 hours with three lots of 5 per cent aqueous pyridine. The extracts were combined and evaporated to small volume. The agar was washed with 95 per cent alcohol and dried. Most of the agar was recovered.

Solution 1 was prepared with 1 per cent Difco agar, 1 per cent agar purified with pyridine, 3 per cent agar purified with pyridine, and 1 per cent purified agar plus the extract equivalent to 1 per cent agar

TABLE 3

EFFECT ON GAMETIC REPRODUCTION OF PURIFYING AGAR WITH METHYL ALCOHOL OR AQUEOUS PYRIDINE. ALL MEDIA CONTAINED DEXTROSE, ASPARAGINE, MINERAL SALTS, AND THIAMIN

Medium	Six inoculations		Two inoculations	
	Progamete production	No. zygotes per plate	Progamete production	No. zygotes per plate
Solution 2 plus				
1% Difco agar	Line 2 mm. wide	6	.....	.....
1% agar purified with methyl alcohol	Thin line	2	.....	.....
methyl alcohol extract equal to 1% agar	Thin line+	1	.....	.....
Solution 1 plus				
1% Difco agar	Line 2 mm. wide	4	None	None
1% agar purified with pyridine	Very few	None	None	None
3% agar purified with pyridine	Line 2 mm. wide	15	Thin line	3
1% purified agar extract equivalent to 1% agar	Line 2 mm. wide	3	None	None

(fig. 5). Plates in duplicate were inoculated at opposite edges with a drop of a spore suspension of the two strains of *Phycomyces;* plates in duplicate were also inoculated with six drops of spore suspension arranged in two lines about 3 cm. apart. The progamete and zygote production was better on the 1 per cent Difco agar than on the 1 per cent purified agar (table 3). Addition of the extract to the purified agar improved the medium but did not make it superior to the 1 per cent unpurified agar. The results on the 3 per cent purified agar were superior to those on the 1 per cent unpurified agar, but not equal to those secured earlier on a 3 per cent unpurified agar.

It appeared, therefore, that the agar contained material favorable to gametic reproduction by *Phycomyces*, and that this material could be extracted in part by dilute methyl alcohol or by aqueous pyridine.

What is this beneficial material? It seems to be organic rather than inorganic, and probably belongs in the class of growth substances (6). Extraction of the agar with pyridine reduced the ash from 3.56 to 2.41 per cent. Addition of agar ash, however, prepared by ashing at low red heat and then heating to dryness with HCl, did not improve growth or gametic reproduction on the purified agar, and was ineffective when added to a 1 per cent agar medium containing

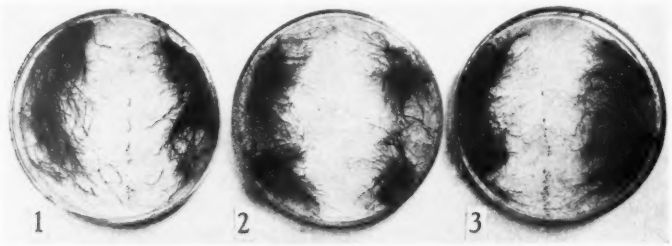

FIG. 5.—Purification of agar and gametic reproduction. All dishes contain 1 per cent agar and same amounts of dextrose, asparagine, mineral salts, and thiamin. *1*, Difco agar; *2*, same extracted with pyridine; *3*, same extracted with pyridine plus extract equivalent to 1 per cent agar. Inoculated in six places. Age 7 days.

thiamin. Increasing the trace elements two and a half, five, or ten times and additions of sufficient zinc to make the total concentration 0.38, 0.75, or 1.5 ppm instead of the 0.15 ppm in the solutions used in this investigation was ineffective. Furthermore, the KH_2PO_4 and $MgSO_4$ employed in making the media were not specially purified and probably contained many trace elements.

EFFECT OF DEXTROSE AND ASPARAGINE

RONSDORF (7) and SCHOPFER (9) found that increased amounts of available nitrogen decreased gametic reproduction of *Phycomyces*. In these experiments also it was observed that the larger amounts of asparagine decreased the number of progametes and zygotes, and sufficiently large amounts (5 or 10 gm. per liter) inhibited them entirely. It appeared that gametic reproduction was interfered with

when either dextrose or asparagine was markedly deficient or was present in large amounts; the asparagine was more effective in this respect than the carbohydrate. From 0.5 to 1 gm. of asparagine per liter and from 10 to 75 gm. of dextrose per liter seemed most favorable.

TABLE 4

AMOUNTS OF ASPARAGINE AND DEXTROSE AND GAMETIC REPRODUCTION OF PHYCOMYCES GROWN ON 1 PER CENT DIFCO AGAR CONTAINING MINERAL SALTS, THIAMIN, AND AMOUNTS PER LITER OF DEXTROSE AND ASPARAGINE GIVEN IN THE TABLE

GRAMS PER LITER		RELATIVE GROWTH	TWO INOCULATIONS		SIX INOCULATIONS	
DEXTROSE	ASPARAGINE		PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE	PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE
50	0.0	Extremely sparse	None	0	Few	4
50	0.1	Very sparse	Scattered line	2	Few	2
50	0.2	Sparse	None	0	Scattered line	1
50	0.5	Medium	None	0	Line 2 mm. wide	4
50	1.0	Medium+	None	0	Line 2–3 mm. wide	26
50	2.0	Heavy	None	0	Line 4 mm. wide	2
1	0.5	Sparse	None	0	None	0
10	0.5	Medium−	Few	0	Line 2 mm. wide	13
25	0.5	Medium	Scattered line	10	Line 2 mm. wide	31
75	0.5	Medium	Few	0	Line 2 mm. wide	10
100	0.5	Medium	Few	0	Line 2 mm. wide	4
50*	0.5	Medium+	Line 3 mm. wide	43	Line 4 mm. wide	63
50†	0.5	Heavy	Line 4 mm. wide	31	Line 6 mm. wide	24

* Potato extract equivalent to 2.75 gm. potato per plate added.
† Equivalent to 13.75 gm. of same.

Because of certain other observations of interest, an experiment in which different amounts of dextrose and asparagine were used will be described in detail.

To a medium containing per liter 10 gm. Difco agar, 0.5 gm. $MgSO_4 \cdot 7H_2O$, 1.5 gm. KH_2PO_4, 0.25 mg. thiamin, and the mineral supplements described earlier, the quantities of dextrose and asparagine given in table 4 were added. Plates in duplicate were inoculated at the edges with drops of a spore suspension of the two opposite strains of *Phycomyces*, and duplicate plates were inoculated also with three drops of each strain in parallel lines about 3 cm. apart. The observations are summarized in table 4.

On the media containing 50 gm. of dextrose per liter and no asparagine growth was extremely sparse, demonstrating that the nitrogen in the agar was insufficient in amount or unavailable for the development of *Phycomyces*. The addition of 0.1 gm. or 0.2 gm. of asparagine improved the growth although it was still scant. With still further increase in asparagine growth increased, the heaviest occurring on the medium containing 50 gm. dextrose and 2 gm. asparagine per liter.

Gametic reproduction also was affected by the amount of asparagine. When the inocula of the two strains were located 3 cm. apart and six were used on each plate, the number of progametes increased with increasing quantity of nitrogen up to 2 gm. of asparagine per liter. The number of zygotes per plate was greatest on the medium containing 50 gm. dextrose and 1 gm. asparagine. With smaller amounts of asparagine the number of zygotes was considerably decreased. On a percentage basis, however, the proportion of zygotes to progametes was highest on the medium to which no nitrogen was added. Although the line of progametes was widest on the medium containing 2 gm. of asparagine per liter, few zygotes were formed, and other observations indicate that a larger amount of asparagine inhibited the formation of progametes also.

When the plates were inoculated in two places about 8.5 cm. apart, a scattered line of progametes and two zygotes per plate were formed on the medium containing 50 gm. dextrose and 0.1 gm. asparagine per liter; no progametes were produced on the other media containing 50 gm. dextrose and different amounts of asparagine.

With asparagine constant at 0.5 per liter growth was sparse on the medium containing 1 gm. dextrose per liter and increased with increasing dextrose up to the medium containing 25 gm. per liter. Amounts of dextrose beyond this point up to 100 gm. per liter appeared to have little effect upon growth. With six inoculations per plate no progametes were formed on the medium with 1 gm. of dextrose and 0.5 gm. asparagine per liter, while the number formed with other amounts of dextrose was about the same no matter whether the dextrose was 10 or 100 gm. per liter. The maximum number of zygotes appeared on the plates containing 25 gm. of dextrose per liter; with amounts above and below this quantity the number of

zygotes decreased. With two inoculations per plate few or no progametes were formed and no zygotes were produced except on the medium containing 25 gm. dextrose and 0.5 asparagine, on which a scattered line of progametes and ten zygotes per plate developed.

It is clear from these results that the amount of dextrose and the amount of asparagine affected gametic reproduction. When either was markedly deficient or present in large amounts gametic reproduction was interfered with. Some progametes and zygotes were formed on the low nitrogen media where growth was severely limited and were not produced on the low sugar medium where also growth was scanty. Furthermore it appears from these results that increasing the dextrose or the asparagine did not increase the formation of progametes and zygotes to the extent observed with additions of extracts of potato, corn meal, or oatmeal. This suggests that the effect of these extracts was not associated with assimilable carbohydrate or nitrogen compounds.

Effect of plant extracts

Schopfer (**8, 9, 10, 13, 15, 16**) found that extracts of various materials of natural origin favored gametic reproduction of *Phycomyces*, but in his experiments the extracts were added to an agar medium which lacked thiamin. In no instance was the effect of an extract upon gametic reproduction determined in the presence of crystalline thiamin. Schopfer's results with extracts of rice polishings, vitamin B concentrate, and other materials appear superior to those he reported for an agar medium supplemented with crystalline thiamin, and he states that he is not certain that the results with the materials of natural origin can be accounted for entirely by their thiamin contents. Nevertheless since thiamin, a necessity for the growth of *Phycomyces*, was a constituent of all the extracts he added to an agar medium lacking that growth substance, it is not possible to assert that the effect of the extracts was not because of their thiamin content. It seemed advisable, therefore, to determine whether extracts of materials of natural origin would affect gametic reproduction when an excess of thiamin was separately supplied. In this way the effect of the thiamin in the natural substances would be eliminated.

Extracts of potato, corn meal, and oatmeal were used since these materials are frequently employed in preparing culture media. The extracts were made by boiling the material with water, or steeping it for a day or two in 50 per cent methyl alcohol. The extracts were decanted, centrifuged, filtered, and concentrated by evaporation. The extract from 0.03 to 12.5 gm. of the original material was added to each petri dish. The experiments will not be described in detail, but the results showed that plant extracts benefited gametic reproduction in the presence of excess thiamin (figs. 1, 4). Their effect therefore appeared to be caused by something other than their thiamin content.

The effect of the agar and plant extracts appeared to be additive; that is, an amount of plant extract which was ineffective or had little effect in a 1 per cent agar medium was decidedly beneficial in the same medium containing 2 per cent agar.

The plant extracts overcame to some extent the inhibitory action of excess nitrogen, or—reversing this statement—the beneficial effect of plant extracts was nullified by increasing the amount of asparagine. For example, no gametes were obtained on a 1 per cent agar medium containing dextrose, mineral salts, thiamin, 5 gm. of asparagine per liter, and the extract of 0.625 gm. of oatmeal per petri dish. When the asparagine was reduced to 1 gm. per liter a line of progametes was produced with the addition of the same quantity of oatmeal extract. No progametes were formed when the oatmeal extract was omitted. These results were obtained with inoculations at opposite edges of the dishes.

The beneficial effect of the plant extracts did not appear to be caused by their content of assimilable nitrogen or carbohydrate. This followed from the observation that an increase in the asparagine or dextrose in the media did not benefit gametic reproduction to the same degree as did the plant extracts (table 4).

SCHOPFER (9) found that the inhibitory action of larger quantities of asparagine could be overcome in part by the addition of impurities extracted from a Kahlbaum maltose, and concluded that the active impurity aids the fungus in resisting the inhibition resulting from the larger quantities of asparagine.

Effect of liquid media

Since agar appeared to contain substances favorable to gametic reproduction of *Phycomyces* at 25° C., an effort was made to find a medium which was free from such beneficial substances. Asbestos cloth heated in a muffle furnace and washed with HCl and distilled water was saturated with solution 1. *Phycomyces* did not grow on this medium. Asbestos cloth covered with a sheet of glass cloth made from glass wool by suitable heat treatment in a muffle furnace was also ineffective as a solid substratum. On a pad of glass wool covered with a sheet of glass cloth and saturated with solution 1 growth was scanty. Since a solid medium of the type desired was not available, a thin layer of liquid medium in a petri dish was used.

Ten or 15 ml. of solution 1 in a petri dish was inoculated with bits of young mycelium of the plus and minus strains of *Phycomyces;* the bits were placed 1–2 cm. apart so that they soon grew together. A few progametes were produced but no zygotes were observed. The same results were secured when the medium in the dishes was inoculated with the spores of the plus and minus strains. These results are in contrast with those on an agar medium made with the same nutrient solution and inoculated with the two strains 1–2 cm. apart. They suggest that *Phycomyces* will not produce progametes or zygotes in any number at 25° C. in a medium of sugar, asparagine, mineral salts, and thiamin. Furthermore, the addition to solution 1 of potato extract induced the formation of hundreds of progametes and zygotes when the liquid medium was in petri dishes and inoculated with bits of young mycelium or spores. Only one nutrient solution was used in these experiments with liquid media. It is possible that with variations in the sugar, nitrogen, and mineral salts, considerable gametic reproduction can be secured at 25° C. in a medium with no supplement other than thiamin, although there is little encouragement for such a possibility from those experiments in which different quantities of asparagine and sugar were used in an agar medium.

Temperature

The preceding experiments were carried out at 25° C. This temperature restricts gametic reproduction by *Phycomyces*. Even on a

favorable medium only a small proportion of the progametes develop into zygotes, and many of the zygotes do not mature. On the other hand vegetative growth is vigorous, and it is more convenient to deal with a small number of zygotes than with the many which form at the lower temperatures.

The effect of temperature on gametic reproduction requires further investigation. It is not likely, however, that a lower temperature of incubation will give results which would materially affect the general conclusions to be drawn from these experiments. At 18°–20° C., distance between inocula and the addition of plant extracts to an agar medium were found to affect the production of zygotes in the same direction as at 25° C. At the lower temperature the number of zygotes is much greater.

Discussion

These results can best be explained by assuming that growth substances in addition to thiamin must be supplied *Phycomyces* if gametic reproduction is to occur to any extent at 25° C., and that these substances have a beneficial effect at lower temperatures also, although less essential there. Other explanations may be offered: (1) that the effective material is inorganic; (2) that the action of the agar and plant extracts is to nullify some injurious action of the medium; and (3) that we are dealing with a general growth effect not assignable to any specific substances. It is recognized that the validity of the assumption that growth substances in addition to thiamin are concerned in gametic reproduction of *Phycomyces* will not be demonstrated conclusively until such substances are actually isolated and their effects determined.

If growth substances are concerned they should not be regarded as reproductive substances in the sense that they are concerned in gametic reproduction only. It seems more likely that they are required for vegetative growth and other phases of development of the fungus also, and are used up more or less in proportion to the extent and rapidity of growth. The unknown substances are synthesized to some extent by the organism, but under some conditions in amounts inadequate for extensive gametic reproduction.

The writer is inclined to assume the activity of growth substances

rather than inorganic substances because the basic media contained numerous trace elements and additions of agar ash, and increased amounts of trace elements were ineffective. On the other hand, all possible elements in various amounts were not investigated. The hypothetical substances would not appear to consist of available nitrogen or carbohydrate because the nitrogenous and carbon compounds in agar are not available to *Phycomyces*. Furthermore, it is possible on such an assumption to explain other results, as follows:

1. Distance between inocula.—The growth substances in the agar are exhausted in the vegetative growth and formation of sporangia if the inocula are sufficiently separated and contact of the two mycelia is delayed.

2. Inhibitory effect of larger amounts of asparagine.—With increased asparagine vegetative growth is increased and the limited amount of growth substances supplied in the agar is exhausted.[5]

3. Correction of inhibitory effect of asparagine by additions of plant extracts.—The plant extracts increase the amount of growth substances available, which are then adequate to meet the needs for the increased vegetative growth and for gametic reproduction.

4. Effect of plant extracts.—The unknown growth substances are present in the plant extracts.

5. Results at 25° C. in a synthetic liquid medium.—The liquid medium lacks the unknown growth substances, which are synthesized by the fungus in amounts adequate for vegetative growth but not, under the conditions employed, in amounts sufficient for gametic reproduction.

6. Effects of temperature.—Either the formation of the hypothetical substances by the fungus is greater, or the requirement is less, at the lower temperatures.

7. If growth substances are concerned they are water-soluble, soluble in dilute alcohol, and thermo-stable.—The results with pure crystalline B_2 (lactoflavine), pantothenic acid, nicotinic amide, synthetic vitamin B_6, carotene, and α tocopherol (vitamin E) have thus far been negative. Biotin (**4**), pantothenic acid, and heteroauxin (**16**) have been reported ineffective with *Phycomyces*.

[5] Such an explanation would not apply to amounts of asparagine large enough to interfere with growth.

Even if these assumptions are correct, it should be emphasized that the substances dealt with are probably not the only ones concerned with gametic reproduction in *Phycomyces*. In none of the media used have sex organs appeared on one strain in the absence of the other; yet BURGEFF (**2**) has shown that substances diffusible through a celloidon membrane from one strain induce development of gametophores in the other. It has already been pointed out (**6**) that growth substances other than thiamin seem to be concerned in the spore germination of this fungus. It would appear that the relation of *Phycomyces* to an external supply of growth substances may be complex and involve more than the single substance, thiamin.

Summary

At 25° C. the intensity of gametic reproduction of *Phycomyces blakesleeanus* upon an agar medium containing dextrose, asparagine, mineral salts, and thiamin was affected by the distance between the inocula of the plus and minus strains, by the concentration of the agar, by the addition of plant extracts, by the amount of asparagine, and to a less extent by the amount of carbohydrate. Gametic reproduction was decreased with the greater distance between the inocula, and by the larger amounts of asparagine; it was favored by increasing agar concentration and by the addition of plant extracts. The beneficial materials in agar could be extracted in part by dilute methyl alcohol or by aqueous pyridine. The addition of plant extracts overcame in part the inhibitory effect of the larger amounts of asparagine. Little gametic reproduction was obtained at 25° C. in a liquid medium containing dextrose, asparagine, mineral salts, and thiamin unless a plant extract was added. It is concluded that growth substances in addition to thiamin are probably concerned in the gametic reproduction of *Phycomyces*.

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EVIDENCE FOR AUXIN PRODUCTION IN ISOLATED ROOTS GROWING IN VITRO[1]

J. VAN OVERBEEK

In an earlier paper it was reported that auxin was found in isolated roots that had been subcultured for 3 weeks (**11**). The question then arose whether auxin is produced in these isolated roots. Experiments designed to answer this question are reported here.

TABLE 1

TOTAL AMOUNT OF AUXIN PER AVERAGE ROOT IN 10^{-6} γ INDOLEACETIC ACID EQUIVALENTS, DURING SUBCULTURING. P_0 INDICATES INITIAL ROOT TIP; P_1, P_2, ETC., INDICATE FIRST CULTURE, SECOND WEEK OF CULTURE (FIRST SUBCULTURE), ETC.

August 2, 1938	P_0	230.0	Sept. 24, 1938	P_2 Tip	48.4
August 9.....	P_1 Tip	75.3	Sept. 30.....	P_3 Tip	50.1
	Base	39.2		Base	38.1
	Total	114.5		Total	88.2
August 16....	P_2 Tip	127.2	October 8.....	P_4 Entire root	36.5
	Base	53.6			
	Total	180.8			

Sufficient peas of the variety Perfection were sterilized and germinated (**3**) so that more than 1000 root tips 3–4 mm. long and each weighing on the average 10 mg. could be excised. About 200 were analyzed for their auxin content in the manner previously described (**8, 9, 11**). The remaining tips were cultured in petri dishes containing a basic nutrient solution to which vitamin B_1 was added (**3**). After one week in culture about 400 roots were analyzed for auxin. The total amount of auxin in these roots (P_1) was smaller than that of the initial tip (P_0), as shown in table 1. Tips 10 mm. long of the remaining P_1 roots were subcultured and maintained for another week. At the end of this week the roots (P_2) had a higher auxin

[1] Work carried out with the aid of the Works Progress Administration, Official Project No. 665-07-3-83, Work Project L-9809.

content than the P_1 tips from which they had originally come. This indicates an auxin production by these roots.

The experiment was continued with another series of roots (P_2) from which 10-mm. tips were taken. These tips contained on the average 48.6×10^{-6} gamma indoleacetic acid equivalents. The roots from these tips after one week contained on the average 88×10^{-6} gamma, thus showing an increased auxin content. The tips of this P_3 series, containing 50.1×10^{-6} gamma of auxin per average root, developed little during subsequent subculturing and contained at the end of the week 36.5 gamma indoleacetic acid equivalents. The results of this experiment indicate, although not conclusively, the possibility of an auxin production in the isolated roots.

Experiments had to be carried out on a larger scale, using an improved nutrient. ADDICOTT and BONNER (1) improved the nutrient solution by adding both vitamin B_1 and nicotinic acid. In this solution pea roots have been subcultured for 34 consecutive weeks. Only the bases were analyzed for auxin and the tips were subcultured. Even after 34 weeks of subculturing a large amount of auxin (1000×10^{-6} gamma indoleacetic acid equivalents per average root base) was found to be present, and there is no reason to believe that later cultures would be devoid of it. In table 2 the results of an analysis covering 20 weeks are given. It should be noted that this table is composed of several experiments which together cover the entire range from 0 to 20 weeks of subculture. No data were available for the 15th, 16th, and 17th weeks; the auxin content at this stage was estimated to be equal to the average of the available data for P_1 to P_{20}. A total amount of auxin 2043×10^{-6} γ indoleacetic acid equivalents was extracted from the bases during the twenty successive weekly transfers (or 1737×10^{-6} γ if the 15th, 16th, and 17th weeks are not counted). The average amount of auxin extracted from the initial root tip (P_0) is 232×10^{-6} indoleacetic acid equivalents. This shows that an auxin amount nine times larger than that present in the initial tip can be extracted from roots grown from this tip. These results were obtained when the bases as well as the initial tips (P_0) were extracted in the usual manner (**8, 9, 11**) by standing in ether for 1–3 days. In a special experiment in which initial tips were extracted in a Soxhlet extractor, an auxin amount as high as

720 × 10^{-6} γ indoleacetic acid equivalents was once found for P_0 (table 2). Thus even if initial tips were exhaustively extracted in a Soxhlet apparatus they still contained only about 30 per cent of the total amount of auxin extracted in the ordinary way from the bases of twenty consecutive subcultures. In view of the fact that P_{34} roots

TABLE 2

TOTAL AMOUNT OF AUXIN PER AVERAGE ROOT IN 10^{-6} GAMMA INDOLEACETIC ACID EQUIVALENTS, DURING SUBCULTURING. EXCEPT FOR P_0, ONLY ROOTS WITHOUT THEIR TIP WERE ANALYZED. S INDICATES EXTRACTED IN A SOXHLET EXTRACTOR; E INDICATES ESTIMATED VALUE

CULTURE	DATE	CONTENT	AVERAGE	CULTURE	DATE	CONTENT	AVERAGE
P_0 (initial tip)	Aug. 2, 1938	230	232	P_8	Apr. 10, 1939	298	298
	May 9, 1939	201		P_9	Mar. 23, 1939	150	
		265			Apr. 10, 1939	152	161
	May 16, 1939	481 S			Apr. 17, 1939	180	
		720 S		P_{10}	Mar. 28, 1939	130	119
P_1	Aug. 9, 1938	39	39			102	
	Aug. 16, 1939	54			Apr. 17, 1939	126	
P_2	Sept. 1, 1938	67	53	P_{11}	Apr. 10, 1939	85	
	Sept. 17, 1938	55			Apr. 17, 1939	85	91
	Apr. 13, 1939	35			Apr. 28, 1939	102	
P_3	Sept. 30, 1938	38	93	P_{12}	Apr. 28, 1939	19	48
	Apr. 17, 1939	147			May 5, 1939	77	
P_4	Apr. 28, 1939	27	40	P_{13}	May 5, 1939	40	40
		47		P_{14}	Mar. 28, 1939	226	226
		52		P_{15}			102 E
		35		P_{16}			102 E
P_5	May 5, 1939	33	40	P_{17}			102 E
		46		P_{18}	Apr. 17, 1939	115	115
P_6	Jan. 24, 1939	145	134	P_{19}	Apr. 28, 1939	78	78
		142		P_{20}	May 5, 1939	131	131
	Mar. 23, 1939	115					
P_7	Mar. 28, 1939	31	31		Total for 20 bases....		2043

extracted in a Soxhlet apparatus yielded 1000 × 10^{-6} γ (see following), it is likely that if the other bases also were extracted in the Soxhlet apparatus this percentage would be considerably smaller.

A small number of roots were left that had been subcultured for 34 weeks. The bases of fifteen such roots, each weighing on the average 18.9 mg., were extracted in the Soxhlet apparatus for 30 hours. The extract was taken up in 0.3 cc. of 1.5 per cent agar and gave a curvature of 7.3° in the *Avena* test. The blank control did not give

any auxin curvature (+0.8°) and a control having 55 γ indoleacetic acid per liter curved 7.1°. From this the average auxin content of the average P_{34} base was calculated and found to be 1000×10^{-6} γ indoleacetic acid equivalents, which is a surprisingly large amount of auxin for roots.

Discussion

These results show that auxin is produced in roots growing in vitro. It seems probable by analogy with coleoptiles, stems, etc., that in these roots auxin also disappears, which may be due either to auxin utilization during growth or to destruction (**12**). Thus the auxin found by extraction may be the balance of auxin produced and auxin lost. It is generally assumed that auxin is produced

TABLE 3

EFFECT OF DECAPITATION ON FORMATION OF SIDE ROOTS
P_2 ROOTS CONTINUED FOR ANOTHER WEEK

	DECAPITATED	INTACT
Average length of main root	55.9 mm.	78.5 mm.
Average number of side roots	3.8	1.3
Average total length of side roots	16.5 mm.	4.3 mm.
Number of roots	42	45

in the root tip because it has been observed that it is given off by the excised tip. In a paper dealing especially with this problem (**10**) it has been shown that the auxin given off by the tips of germinating pea roots is not produced, but is present in the tip when the latter is cut from the root. The fact, therefore, that auxin in relatively large amounts is given off by excised root tips of germinating peas cannot be construed to mean that auxin is produced by such tips. Another argument which has been used in favor of auxin production in the root tip is that a higher concentration is found in the tip than elsewhere in the root (**4, 6, 11**). This, however, may be due to an auxin accumulation in the tip (**6**). The following experiment tested this point.

Roots at the end of the second week of subculturing (P_2) were decapitated and put back in fresh nutrient solution for a further week. As shown in table 3, decapitation greatly promotes the development of side roots (**7**). At the end of the week the roots were divided into two groups according to their having developed side

roots or their failure to do so. Table 4 shows that the original base weighed on the average 11.4 mg. At the end of the week the 192 roots which had developed side roots weighed on the average 23.2 mg., whereas the 198 roots which failed to develop side roots weighed 10.9 mg., which is approximately the same as the original base. The auxin content of the original base was 66.6×10^{-6} γ on the average. After one week the roots which had developed side roots contained 27.8×10^{-6} γ per root and those without side roots 86.4×10^{-6} γ indoleacetic acid equivalents (average 57.1). This was rather the reverse of what could have been expected on the basis of an increased auxin production due to an increased number of new root tips. It should be remembered, however, that the auxin extracted gives only the balance of auxin produced and lost and that the latter factor

TABLE 4

AUXIN CONTENT IN 10^{-6} GAMMA PER AVERAGE ROOT DURING DEVELOPMENT OF SIDE ROOTS

Decapitated P_2 roots (11.4 mg.)	66.6
One week later:	
192 with side roots (23.2 mg.)	27.8
198 that failed to develop side roots (10.9 mg.)	86.4

probably was larger in the case where side roots were formed on account of their faster growth. On the other hand, AMLONG (**2**) reported evidence indicating that if the root tip of germinating *Vicia* is removed the auxin content of the remaining root is decreased. NAGAO (**5**) recently reported that if excised root tips were placed on nutrient agar they began to grow and produce auxin. VAN OVERBEEK (**10**) showed that the auxin which diffuses out of the excised root tips into dextrose agar is present when the tip is cut off.

A factor of uncertainty in this type of experiment with roots is the question of the possible presence of an inactive auxin precursor. Since we cannot as yet test quantitatively for this factor, we do not know how much of it is present in the initial root tip. Neither do we know how easily this substance is converted into active auxin. One could imagine that in pea roots, even under the condition of ether extraction, an equilibrium exists between auxin precursor and active auxin. This could explain why Soxhlet extraction, where roots and extract are separated, yields more auxin than ordinary extraction.

From the experiments presented here it can be concluded that auxin is produced in isolated pea roots grown in vitro under sterile conditions. This may be due to either one or both of two things: An active synthesis of auxin from the nutrient medium (inorganic salts, sugar, thiamin, nicotinic acid) or the gradual activation of an auxin precursor present in the initial tip and "carried along" during subculturing.

Summary

1. Evidence is presented which shows that auxin is produced in isolated pea roots cultivated in vitro under sterile conditions. The roots were cultured in such a way that at the end of each week the culture was continued with the 10-mm. tip only. The basal parts of the roots, that grew approximately 700 mm. per week, were discarded. The total amount of auxin extracted from these discarded bases during 20 weeks of culture was nine times as much as that extracted from the initial tip from which the cultures originated.

2. Even at the end of 34 weeks of culturing, roots were found to contain as much as 1000×10^{-6} γ indoleacetic acid equivalents of auxin per root.

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VERNALIZATION OF MARQUIS WHEAT AND OTHER SPRING CEREALS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 507

D. J. WORT

(WITH SIX FIGURES)

Introduction

In 1933, WHYTE and HUDSON (22) presented a translation and compilation of the literature on vernalization of plants. A bulletin (9) appeared in 1935 which summarized the research on this subject in the Soviet Union and twenty-six other countries. Many aspects of vernalization, theoretical and practical, have recently been discussed by WHYTE (21).

There is great diversity of opinion with respect to the reaction of spring cereals to vernalization. Data collected by FOKEEV and VYROV (3, 4) from various places in the U.S.S.R., where extensive use of vernalization has been made, showed 2 to 4 days earlier emergence of vernalized seedlings from the soil. Vernalization stimulated root development, which in turn led to increased development of green parts. The thicker stand, which was maintained throughout the vegetative season, reduced weed growth. At harvest vernalized plants were on the average 9.5 cm. taller. Vernalized grain began earing 2.5 days earlier than controls. In dry districts there was a recorded increase in grain yield of 28.6 per cent as compared with 17–18 per cent in other districts. The quality of the wheat grain was also improved. ZENJUK (24) grew vernalized wheat at the Minsk Marshland Station to determine the acceleration of maturity. In later sowings (May 3) vernalization accelerated the onset of all developmental phases, whereas in early sowings the difference was evident only prior to ear emergence.

VAN HOEK (20), working in Holland, vernalized spring wheat for 7 days and for 16 days, at 3°–6° C. The 16-day treatment was the more successful, resulting in an acceleration of earing of 3 days and of maturity of 1–4 days. GMELIN (6), also working in Holland and

using the same variety of wheat as VAN HOEK, could obtain no acceleration of maturity. In Rumania, PIESCU (16) found that vernalized spring varieties headed at the same time as controls.

In the United States, MCKINNEY *et al.* (15) grew vernalized Marquis wheat at Langdon, North Dakota, 20 miles south of the Canadian boundary. No decreases in time to mature were obtained, but treatment for 10 days gave average increases in yield of 19.07 per cent over unchilled, ungerminated controls. TAYLOR and COFFMAN (19) vernalized Isogold, a variety of spring oats. Treatment retarded heading by 2 days and gave a decrease of 37 per cent in yield. This decrease is ascribed to less germination, since the stand in the vernalized plot for a period of 4 years averaged only 51 per cent of that in the untreated plots.

Referring to experimental data, KONSTANTINOV (10) asserts that although vernalization of cereals, maize, millet, sunflower, Sudan grass, and other crops is widely used in all parts of the Soviet Union, the efficacy of vernalization is closely connected with the region and after-sowing conditions. The cold spring of southeast Russia is rather unfavorable for vernalized sowings, or at least less favorable than the warm, moist, prolonged spring of certain other regions. The efficacy of vernalization varies also with the time of sowing, later sowings being more beneficial.

The chief edaphic difference between the early and late plantings, particularly at high latitudes, would be soil temperature, which would show an increase as the spring advanced. The preceding results therefore point to a possible correlation between soil temperature and efficacy of vernalization.

The phasic development theory assumes that plants can enter upon the next stages of development, given appropriate conditions, only when the previous stage has been completed. It is therefore of special importance to be able to determine whether a certain stage has been completed. Assuming that vernalization produces a change in the physico-chemical state of the protoplasm, BASSARSKAJA (1, 2) was able to detect the completion of the thermo-stage by the precipitation of Prussian blue within the tissues of the vernalized embryo upon addition of ferric chloride and potassium ferrocyanide. RICHTER (18) and GAVRILOVA (5) have used a staining technique

based upon the displacement of the iso-electric point of the tissues of the embryo to determine completion of the thermo-stage in cotton and wheat. A modification of the microchemical technique used by these investigators was employed in the diagnosis of vernalization of Marquis wheat.

In the following experiments, which were begun in 1934, the reaction of vernalized spring wheat, oats, barley, and rye with respect to vegetative period, yield, photoperiodic response, and growth at various soil temperatures was investigated. A preliminary attempt to use microchemical methods to diagnose the vernalized state is also described.

General method and terminology

Methods of vernalization are based primarily on the researches of LYSENKO (13). The general procedure for vernalization of all cereals consists in germinating the seeds for 24 hours at 15°–20° C. and then transferring the germinated grain to low temperatures for a period, allowing the young plant to continue development without increasing in size.

For convenience a formula is employed to express the combinations of factors employed in the vernalization treatment. The factors and their units are: (1) length of time of chilling in days; (2) temperature during chilling in degrees Centigrade; (3) amount of water added in percentage of original air-dry weight of the seed. For example, the formula 8:3:60 means that the seed was chilled for 8 days at 3° C. following germination, the water added being 60 per cent of the original air-dry weight of the seed.

The vernalization method used consists of four parts:

1. The amount of water was added to the seeds in petri dishes.
2. The imbibed seeds were allowed to germinate until radicles had pierced the seed coats of a few grains (usually within 24 hours at 15°–20° C.).
3. The germinated seeds were placed in the chilling chamber for the number of days indicated.
4. The covers of the petri dishes were removed and the seeds aerated for a few seconds each day.

Any departures from this method, necessary in the treatment of large quantities of seed, are noted in the text.

Investigation

Effect of vernalization on length of vegetative period and yield

A. Field experiments

Investigations covering the period 1934 to 1937 were conducted at Saskatoon, Saskatchewan (52° N lat., 106° W long.), while the work of 1939 was performed at Chicago, Illinois.

TABLE 1

Treatment, harvest data, etc., for field experiments, 1934–1939

Year	Grain	Vernalization formula	Weight of seed treated	Area sown	Treatment of controls	Acceleration of flowering (days)	Acceleration of ripening (days)	Increased yield due to vernalization	Date of planting
1934	Marquis wheat	5	30 gm.	Five rows 4′ long	Unchilled, ungerminated	See table 2			May 5
		7	"	"	"				"
	O.A.C. barley	13	"	"	"				"
	Hannchen barley	16 : 3 : 33	"	"	"				"
		17							
	Banner oats	18	"	"	"				"
	Victory oats	23	"	"	"				"
1935	Marquis wheat	12:3:33	300 lb.	5 acres	"	0	0	21.04%	May 6
1936	Marquis wheat	12:3:33	60 lb.	1 acre	"	0	0	14.04%	May 7
1937	Marquis wheat	12:3:33	60 lb.	1 acre	(*a*) Unchilled, ungerminated	.....	.....	.....	May 9
					(*b*) Germinated	.....	.....	.....	May 9
	Banner oats	12:3:33	20 lb.	½ acre	As for wheat, 1937	.....	.....	.....	May 9
1939	Marquis wheat	8:3:60	20 gm.	Seven rows 5′3″ long	Germinated	2.3	.....	.....	July 3

Chilling: 1935, in room cooled by snow; others by mechanical refrigeration.
Planting: 1934, 1939, by hand; others by seed drill.
Location: 1939, in Chicago; others in Saskatoon, Sask.

The formula used during the years 1934 to 1937 was that of Lysenko, with respect to amount of water added and temperature during treatment. Various lengths of treatment (table 1) were used in 1934 in an attempt to determine the optimum combination of the several vernalization factors used. The formula used in 1939, 8:3:60, was the one developed in 1938 which gave the greatest acceleration of flowering of Marquis wheat grown in the greenhouse during that year.

A modification of the method just given was necessary to vernalize large quantities of seed. The water was added in three equal amounts, 4 hours apart, and the seeds were stirred periodically to insure uniform moistening.

In 1934 the chilling chamber was a basement room, snow serving effectively to maintain an average temperature of 3° C. During chilling, the grain, which had been spread in a layer 6 inches deep on the floor, was mixed thoroughly once a day to insure adequate aeration. The 60-pound and 20-pound lots treated in 1935, 1936, and 1937 were chilled in bags which allowed a relatively free access of air. In all cases the treated grain was sown, without being dried, on the day it was removed from the refrigeration room. When more than one formula was used all treatments were concluded at the same time.

When the seed drill was used, setting of the drill at 2 bushels per acre and 1 bushel per acre sowed equal dry weights of vernalized and ungerminated, unchilled control grain, respectively. The setting had to be increased slightly beyond 2 bushels in the case of the germinated control.

In the 1935 and 1936 experiments the plots were sampled by harvesting 50 areas of 1 square yard each, taken in regular order throughout the plots. The field plots of treated and control grain were one-quarter mile long and located side by side. The soil conditions in the plots were practically identical.

The experiments of 1934 to 1937, in which 33 per cent water was added to the original dry weight of the seed, gave little acceleration of maturity of Marquis wheat, Banner and Victory oats, and O.A.C. barley, regardless of duration of treatment. In the case of Hannchen barley, however, 5:3:33 and 13:3:33 both resulted in a 7-day advance in flowering (table 2). The formula 8:3:60, used in 1939, accelerated flowering of Marquis wheat in the field by an average of 2.3 days.

Increases in yield were general, amounting to 100 per cent over control in the case of Banner oats, vernalized according to the formula 23:3:33, in the small-scale trial of 1934. The large-scale sowings of 1935 and 1936 gave yield increases by vernalized Marquis wheat of 21.04 and 14.04 per cent, respectively, over the untreated controls. In 1935 the vernalized Marquis wheat showed an increase

in height of 7.2 per cent, an increase in number of fertile spikelets per head of 5.0 per cent, an increase in average kernels per head of 2.5 per cent, and an increased 1000-grain weight of 18.64 per cent.

Drought during the spring and summer of 1936 resulted in a smaller increase in yield due to vernalization than in 1935, when growing conditions were more favorable. Vernalized Marquis wheat did not appear to have any greater or less resistance to the extreme drought of 1937, which caused complete crop failure. In 1939 a week

TABLE 2

ACCELERATION IN DAYS TO FLOWER, TO RIPEN, AND INCREASED YIELD DUE TO VERNALIZATION. FIELD EXPERIMENT, 1934

Days vernalized	Acceleration in days to flower over control					Acceleration in days to ripen over control					Percentage increased yield over control				
	Marquis wheat	O.A.C. barley	Hannchen barley	Banner oats	Victory oats	Marquis wheat	O.A.C. barley	Hannchen barley	Banner oats	Victory oats	Marquis wheat	O.A.C. barley	Hannchen barley	Banner oats	Victory oats
5	2	1	7	−4	0	0	−1	5	−2	1	20.8	64.3	74.3	70.4	50.0
7	−3*	−1	6	−1	−2	−1	−1	2	0	−1	14.6	52.2	37.3	59.0	59.3
13	0	1	7	1	0	0	−1	5	0	1	5.2	54.0	46.1	85.9	31.2
16	−3	1	1	1	−1	−1	−1	4	0	−1	−3.1	3.2	20.4	70.3	28.1
17	−3	0	5	0	−1	−1	−1	2	−1	0	7.3	31.9	2.2	48.1	18.8
18	3	−1	6	1	−2	0	−1	2	0	−2	9.4	51.2	29.6	62.9	25.0
23	2	0	3	−1	0	0	−1	2	−1	−1	27.1	74.1	37.3	100.0	28.1

* Minus sign indicates retardation in flowering or ripening.

of hot dry weather immediately following planting reduced germination of vernalized seeds of Marquis wheat 28.5 per cent more than the reduction in germination suffered by the control grain. At the end of this week the plots were watered and watering was continued periodically throughout the experiment. In spite of the greater reduction in germination, the vernalized plants responded to irrigation by producing 47.5 per cent more heads than controls in 87 days, the period of the experiment.

B. GREENHOUSE EXPERIMENTS

Experiment 1.—The results of the experiments concerning the photoperiodic response of various vernalized cereals, described in a later section, indicated the necessity for developing a formula for treatment, specific for each cereal. An attempt was made at Chicago

during the summer of 1938 to determine the vernalization formula for Marquis wheat which would give the shortest vegetative period. The experiment was begun June 21 and terminated August 26.

The possible combinations, 125 in all, of the following vernalization factors were employed:

1. Length of vernalization period: 5, 8, 11, 14, 17 days.
2. Temperature during vernalization: 3°, 5°, 7°, 10°, 13° C.
3. Water added: 20, 30, 40, 50, 60 per cent.

Two complete series of these various combinations were run as indicated in the following paragraphs. Forty-gm. lots of Marquis wheat were treated, using the procedure outlined at the beginning of this report and the 125 combinations of factors just given.

Series A consisted of lots of seed started at the same time and removed from the mechanical refrigerators at the end of 5, 8, 11, and 14 days. The removals and plantings were on June 27, June 30, July 3, July 6, and July 9. Series B consisted of lots of seed placed in the refrigeration chambers at such times that all the periods of treatment, including 17 days, were completed on July 9. Thus only series B included seed vernalized for 17 days.

Three plants were grown in 6-inch pots filled with loam soil. The control was grain to which water equal to 40 per cent of the dry weight had been added and allowed to germinate 24 hours at 20° C. No control over the temperature of the greenhouse was attempted. This rose nearly every day to 100° F. and fell at night to 70° F.

1. Effect of drying seed subsequent to treatment.—The percentage germination of the grain prior to vernalization was 94.75 and immediately after treatment 95.0. After being air-dried for 3 days following vernalization the germination was 86.0 per cent. Drying the vernalized grain decreased germination by approximately 9.5 per cent.

2. Length of vegetative period.—In table 3 only those combinations of length of vernalization, temperature, and percentage water added which resulted in a decrease in length of the vegetative period, compared with controls, in both series A and B are given. No results complying with this condition were obtained with 14-day treatment although decreases in time to flower were obtained in several cases in series A.

The combination of vernalizing factors which gave the earliest flowering of Marquis wheat under greenhouse conditions was 8:3:60. This formula gave an acceleration of 24 days in series A and 18 days in series B, or an average of 21 days in comparison with controls. In both cases the grain was ripe (the head showing no green color) or would have ripened in 50 days after planting. The formulas 8:10:50 and 8:13:20 produced an acceleration in flowering of 14.5 and 13

TABLE 3

COMBINATIONS OF VERNALIZING FACTORS GIVING DECREASES IN VEGETATIVE PERIODS IN SERIES A AND B, 1938, GROWN IN GREENHOUSE

Vernalization formula	Series A						Series B					
	Days for treated plants to			Control*		Acceleration in days to flower due to vernalization	Days for treated plants to			Control*		Acceleration in days to flower due to vernalization
	Head	Flower	Ripen	Head	Flower		Head	Flower	Ripen	Head	Flower	
5:3:50	39	42	60	47	47	5	41	41	.....	48	49	8
5:5:50	43	46	.....	47	47	1	40	40	.....	48	49	9
5:13:60	43	45	60	47	47	2	30	32	.....	48	49	17
8:3:50	43	44	.....	55	56	12	40	41	.....	48	49	8
8:3:60	31	32	50	55	56	24	30	31	†	48	49	18
8:10:40	47	48	.....	55	56	8	33	33	.....	48	49	16
8:10:50	44	44	.....	55	56	12	30	32	.....	48	49	17
8:13:20	45	46	.....	55	56	10	30	33	.....	48	49	16
8:13:60	48	48	.....	55	56	8	41	41	.....	48	49	8
11:3:30	48	48	.....	52	53	5	33	34	.....	48	49	15

* No controls ripe August 26, when experiment was terminated.

† Nearly ripe in 48 days.

days, respectively; 17:5:60 and 17:7:40 gave an acceleration of 17 days in flowering compared with their controls, in the one attempt using 17-day vernalization. A decrease in temperature during treatment apparently can be counteracted by an increase in moisture content of the seed, within limits.

Experiment 2.—Marquis wheat was vernalized using the formula 8:3:60, developed in the preceding experiment, and grown in 8-inch pots in the laboratory at Saskatoon. The natural daylight was supplemented by electric lights of 200 watts, suspended 12 inches above the plants, to give a total of 15 hours of light per day. Soil temperature readings were taken twice a day, at 9 A.M. and 4 P.M. The average morning reading was 15° C. and the average afternoon soil

temperature was 20° C. The date of sowing was October 23. The number of vernalized plants was twenty-four and controls twelve.

The vernalized plants (*a*) emerged 2 days earlier; (*b*) formed each of the seven leaves at least 2 days earlier; (*c*) headed an average of 5.5 days earlier; (*d*) flowered an average of 7 days earlier; (*e*) ripened 4 days earlier; and (*f*) had a 1000-grain weight 10.37 per cent greater than the controls.

Experiment 3.—The formula 8:3:60 was used to vernalize Marquis wheat at Chicago during the summer of 1939. Three vernalized plants were grown in each of eighteen 6-inch pots and three control plants in each of twenty-one pots.

The wheat, the previous history of which was unknown, did not respond to treatment as did that of the previous year. In the term of the experiment, July 3 to September 3, 46.4 per cent of the treated plants had flowered in an average of 53 days. Of the controls 44.4 per cent flowered in an average of 53.5 days. Treatment increased the number of flowerings but accelerated maturity by only 0.5 day.

In a repetition of the experiment begun August 3, the vernalized grain flowered in an average of 49.7 days and the control in 52.3 days. The acceleration of flowering resulting from vernalization was 2.6 days. In both the 1938 and 1939 experiments the flowering time was shorter and the effects of vernalization more pronounced in later plantings.

The acceleration of flowering caused by vernalization of from 8 to 18 days in 1938 and but 0.5 to 2.6 days in 1939 when two different samples of vernalized Marquis wheat were grown in the greenhouse under similar conditions is perhaps due to differences in the histories of the wheats prior to treatment. The wheat used in 1939 may have been naturally vernalized to some extent during ripening and thus did not respond to the same degree to further vernalization. The result of the 1939 experiment suggested the problem of vernalization of spring wheat prior to reproductive maturity, which is being investigated at the present time.

Photoperiodic response of vernalized spring cereals

Many workers in photoperiodism have determined that some cereals respond to increased photoperiods by an acceleration in

flowering. An experiment was conducted in 1934 to test the comparative response of vernalized and unvernalized spring cereals to continuous illumination.

Marquis wheat, Banner oats, O.A.C. barley, and Prolific rye were vernalized for periods of 5, 7, 13, 16, 17, 18, and 23 days using a temperature of 3° C. and 33 per cent added moisture. Five seeds of each set of cereals were planted in each of two 10-inch pots, filled with loam soil. The plants were grown in a greenhouse the temperature of which was uncontrolled. One complete set (two pots of each

TABLE 4

RESPONSE OF VERNALIZED AND UNVERNALIZED SPRING CEREALS TO INCREASED PHOTOPERIOD

GRAIN	VERNALIZATION FORMULA	DAYS TO FLOWER						ACCELERATION DUE TO VERNALIZATION			DECREASE IN DAYS TO FLOWER DUE TO CONTINUOUS ILLUMINATION	
		GREENHOUSE				FIELD		GREENHOUSE		FIELD		
		VERNALIZED 24 HOURS LIGHT	CONTROL 24 HOURS LIGHT	VERNALIZED NORMAL LIGHT	CONTROL NORMAL LIGHT	VERNALIZED	CONTROL	CONTINUOUS LIGHT	NORMAL LIGHT		VERNALIZED	CONTROL
Marquis wheat	23:3:33	36	37	46	50	59	61	1	4	2	10	13
Banner oats	13:3:33	35	36	46	49	67	68	1	3	1	11	13
O.A.C. barley	13:3:33	35	35	54	58	65	66	0	4	1	19	23
Prolific rye	13:3:33	29	29	36	40	.....	.....	0	4	.....	7	11

cereal for each vernalizing formula) was exposed to normal illumination and another complete set to continuous illumination. Daylight was supplemented by electric lights of 500 watts suspended 4 feet above the tops of the pots.

The plantings, seven in all, were completed on May 5, 1934. Two controls were used, one of seeds germinated for 24 hours at 15° C. and the other of ungerminated, unchilled seeds. The experiment was conducted at Saskatoon.

In all cases the germinated and ungerminated controls flowered simultaneously. TAYLOR and COFFMAN (19) report a similar result in their experiments with spring oats.

The treatments which gave the greatest acceleration in time to flower (extrusion of anthers), compared with controls grown in the same photoperiod, are given—together with flowering data—in

table 4. Data from the field experiment of 1934 are included for comparison. The 13:3:33 treatment resulted in the shortest vegetative period for Banner oats, O.A.C. barley, and Prolific rye. Marquis wheat, however, flowered in the shortest time when vernalized according to 23:3:33.

FIG. 1.—*A:* O.A.C. barley grown in normal illumination. Left to right: control 18 days; vernalized 18 days; control 7 days; vernalized 7 days. *B:* Banner oats grown in normal illumination. Left, control; right, vernalized 13 days.

In each case the acceleration of flowering caused by vernalization, compared with controls grown under the same light conditions, was less in continuous light than in normal illumination. Untreated plants reacted more than treated plants to increased illumination. The cereal whose flowering was accelerated most (19 days when vernalized, 23 days when untreated), due to a photoperiod of 24 hours, was O.A.C. barley; the least responsive was Prolific rye. Other responses are given in table 4.

Grains were produced very sparingly, in some cases having as few as six kernels a head. An estimation of possible yield, however, could be obtained from the size and number of heads. Vernalization resulted in greater height, greater stem diameter, larger average num-

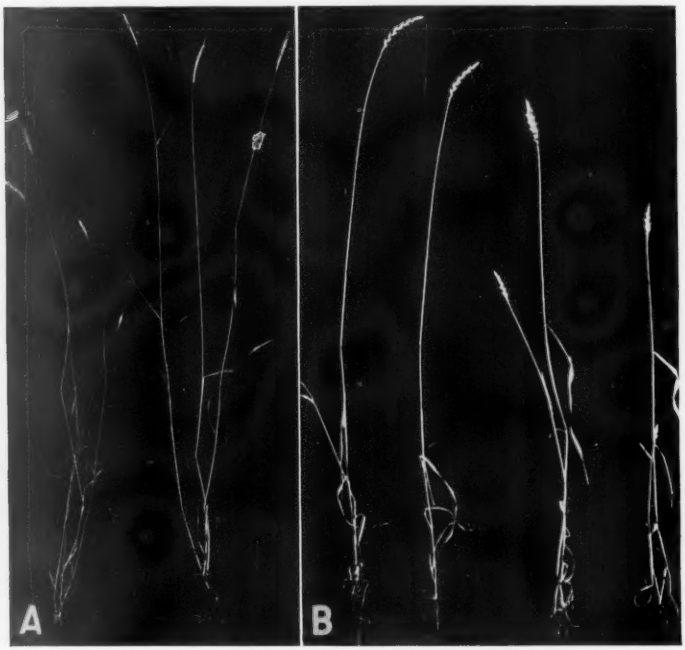

FIG. 2.—*A:* prolific rye grown in normal illumination. Left, control; right, vernalized 16 days. *B:* Marquis wheat. Left to right: vernalized 18 days, grown in continuous illumination; control 18 days, grown in continuous illumination; vernalized 18 days, grown in normal illumination; control 18 days, grown in normal illumination.

ber of tillers, and larger heads than controls when grown under both normal and continuous illumination (figs. 1, 2). Plants receiving 24 hours of light were taller than those grown under normal illumination, the increased height being due to lengthened internodes.

A comparison of time for flowering in the field and in the greenhouse under the same photoperiod indicates, for all cereals grown, that vernalization accelerated maturity more when the plants were

grown at the higher temperature of soil and air in the greenhouse. This possible correlation between response to vernalization and soil temperature was investigated in the experiments described in the next section.

Soil temperature in relation to vernalization

To determine whether a correlation existed between soil temperature and vernalization response, vernalized Marquis wheat was grown at Chicago at various soil temperatures during 1937, 1938, and 1939. The method for each year is given in table 5.

TABLE 5

Methods for growth of vernalized wheat at various soil temperatures

Year	Vernalization formula	Planted	Harvested	Initial growth	Soil temperature (°C.)	Replicate pots, each temperature	Total plants, each temperature	Duration of experiment (days)
1937.....	11:3:40	July 1	Aug. 27	Five days at 26° C., then at temperatures given	22°, 33°, 44°	3	15	57
1938.....	8:5:40	July 9	Aug. 26	Given temperatures from the start	22°–44° in 2° intervals	3	15	48
1939.....	8:3:60	July 3	Sept. 11	As 1937	22°, 26°, 30°, 34°, 38°, 42°	5	15	70

The vernalized and control wheats were planted in loam soil in 2-gallon porcelain containers. Five plants were allowed to grow in each pot during the 1937 and 1938 experiments and but three in 1939. The containers were immersed to within 2 inches of their tops in water. The temperature of the water was thermostatically controlled with the exception of the tank at 22° C. The lowest temperature that could be obtained by using continuously running water from the water mains was 22° C., and the temperature of the water (and hence of the soil in the containers) rose at times to 24° and fell to 21° C. In the other soil temperature tanks the fluctuations were ±0.5° C.

The temperature of the air in the greenhouse was uncontrolled and rose nearly every day to at least 100° F., falling at night to about

70° F. The plants were given sufficient water once a day to keep the soil in all pots moistened to approximately the same degree. More water had thus to be added to the pots at higher temperatures than at lower temperatures. Drains from the containers provided for removal of excess water.

Results of the three experiments were very similar, and only data for 1939 are tabulated in table 6. Significant differences are mentioned in the text.

TABLE 6

HARVEST DATA, SOIL TEMPERATURE EXPERIMENT, 1939

Soil temperature °C.	Vernalized or control	No. plants harvested	Average dry weight (gm.)			Top/root ratio	Greatest average height (in.)	Greatest average root length (in.)	Average no. of tillers/plant	Total heads in 70 days	Average heads per plant	Time for first flowering in days	Average time for flowering in days	Acceleration of flowering due to vernalization in days
			Tops	Roots	Total									
22	V	15	1.45	0.50	1.95	2.9	25.3	17.3	4.7	7	0.47	65	67.6	−1.3
	C	15	1.61	0.40	2.01	4.0	25.0	12.2	4.3	6	0.40	63	66.3	
26	V	15	1.10	0.21	1.31	5.5	22.7	12.1	3.7	12	0.80	48	59.8	4.3
	C	13	0.96	0.23	1.19	4.2	22.5	12.1	3.5	10	0.77	60	64.1	
30	V	12	0.38	0.07	0.45	5.4	19.6	10.1	2.5	3	0.25	52	58.3	2.3
	C	10	0.62	0.11	0.73	5.6	20.1	9.9	2.3	11	1.10	54	60.6	
34	V	14	0.29	0.04	0.33	7.2	16.3	5.4	1.9	6	0.43	48	53.0	2.0
	C	14	0.21	0.03	0.24	7.0	16.4	4.4	1.6	4	0.30	51	55.0	
38	V	15	0.15	0.03	0.18	5.0	12.4	3.3	1.4	4	0.27	52	55.5	6.5
	C	14	0.14	0.03	0.17	4.7	11.5	3.3	1.3	3	0.21	62	62.0	
42	V	8	0.10	0.028	0.128	3.6	10.9	2.9	1.3	.....	.....	.....	.....	.....
	C	8	0.06	0.013	0.073	4.6	7.2	2.3	1.4	.....	.....	.....	.....	.....

As a rule the average height of the vernalized grain, as measured from the soil level to the tip of the longest leaf of the leading tiller, was slightly greater than controls at all soil temperatures throughout the growing period. Figure 3 expresses graphically the average growth of treated and untreated wheat from the age of 5 days to 35 days at the various soil temperatures. The vernalized plants were 3.7 inches taller than the controls at 42° C. and showed a greater dry weight. Less dying of leaves occurred in the treated plants. In the 1937 experiment all control plants grown at 44° C. died within 35 days, but four vernalized plants survived the 57 days, and two headed

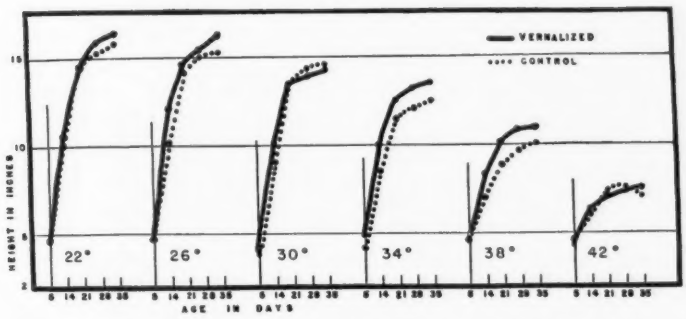

FIG. 3.—Average growth of vernalized and control Marquis wheat at soil temperatures of 22° to 42° C., from age of 5 to 35 days.

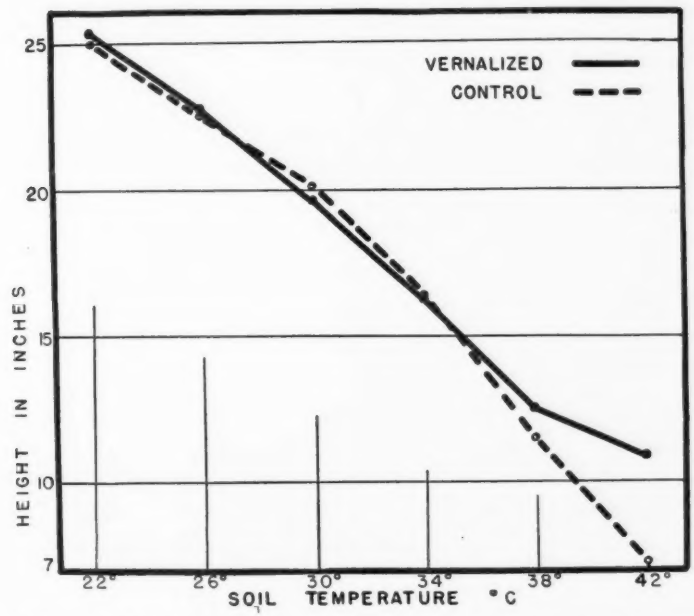

FIG. 4.—Heights of vernalized and control Marquis wheat grown at soil temperatures of 22° to 42° C., at age of 70 days.

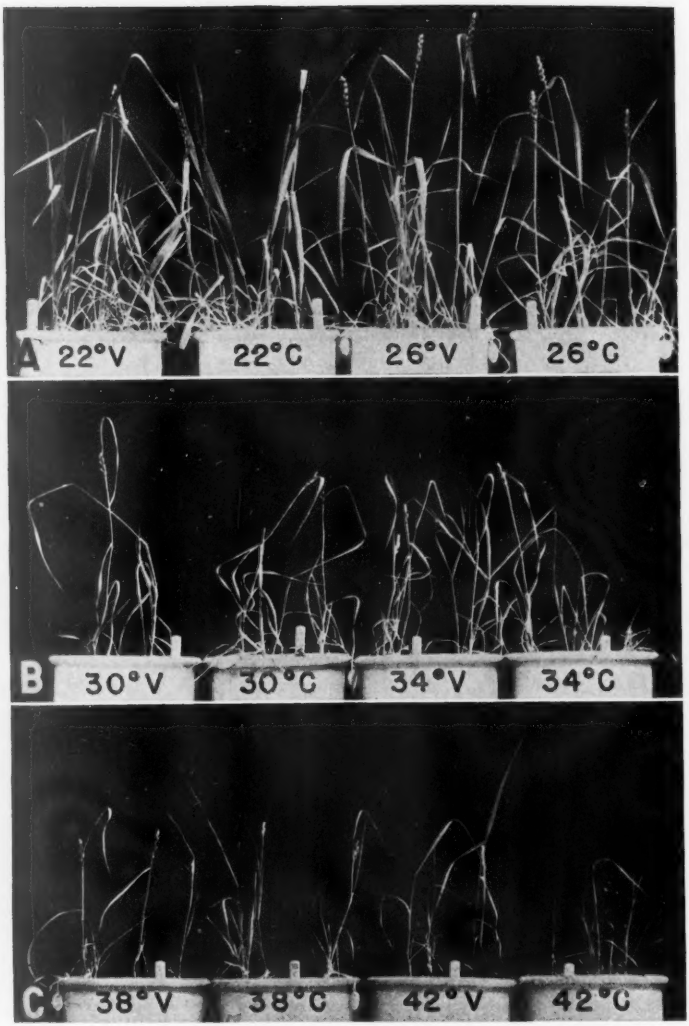

FIG. 5.—Representative plants of vernalized and control Marquis wheat grown at soil temperatures of 22° to 42° C., at age of 70 days. *V*, vernalized; *C*, control.

and produced grain. The height of the plants at 70 days, when they were harvested, is shown in figure 4, while figure 5 shows representative plants from each of the six soil temperatures.

The average greatest root length of treated plants was also greater than that of controls at all temperatures, although the comparative extent of the root systems varied somewhat. Roots decreased in length and extent with increasing soil temperatures.

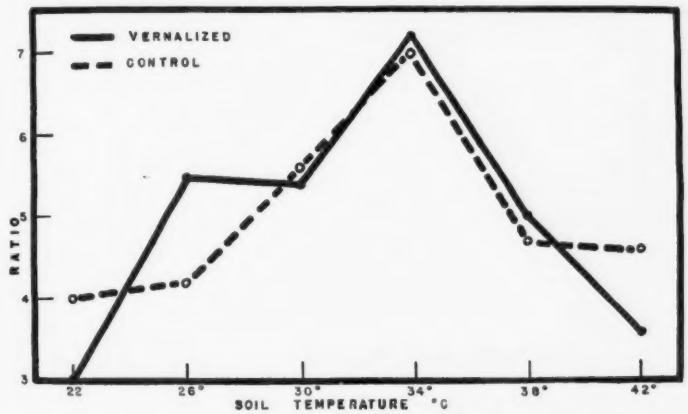

FIG. 6.—Top/root ratics of vernalized and control Marçuis wheat grown at soil temperatures of 22° to 42° C., based upon dry weights at age of 70 days.

The number of tillers was greater on vernalized than on untreated plants, the average increase in the three experiments being 5 to 18 per cent.

No definite statement can be made from the results obtained as to the comparative dry weight behavior of the treated and untreated plants. Dry weights of both were less as temperatures increased. In both vernalized and unvernalized plants there was a rise in the top/root ratio as the temperature increased from 22° to 34° C., followed by a fall at higher temperatures (fig. 6). The high ratio indicates that the tops suffered comparatively less than the roots as the temperature rose to 34° C., but above this temperature the reverse is true.

The flowering behavior in 1937 and 1939 was similar. In 1937 the

control and vernalized plants flowered simultaneously in an average of 57 days when grown at a soil temperature of 22° C. At 33° C. the vernalized plants flowered in 48 days, the controls in 51 days. Two of the vernalized plants that survived at 44° C. flowered in 39 days. All control plants had died within 35 days. In 1939 both control and treated plants flowered in a shorter time as the soil temperature rose from 22° to 34° C. (table 6). Above 34° C. the flowering time was increased or earing prevented. The greatest acceleration of flowering resulting from treatment (6.5 days) occurred in plants grown at a soil temperature of 38° C.

In 1938 the vernalized plants flowered simultaneously at all soil temperatures from 22° to 40° C. The average time was 46 days. The controls had not headed within 48 days, and judging from appearance, would not have done so until at least 55 days. Vernalization accelerated maturity at all temperatures by approximately 10 days. A greater number of flowerings occurred, in the duration of the experiment, among vernalized plants grown at soil temperatures between 34° and 40° than at temperatures of 22° to 32° C.

The flowering behavior thus showed earlier flowering of both vernalized and control plants as the soil temperatures increased from 22° to 34° C.; and greater acceleration of flowering of vernalized plants over controls, or a greater number of flowerings in a given period, at higher soil temperatures.

The survival and flowering of some of the vernalized plants at a soil temperature of 44° C., their greater dry weight and smaller amount of dying of leaves at high temperatures than controls, indicate that—given adequate moisture—vernalized plants withstand high soil temperatures to a greater degree than untreated plants.

Diagnosis of the vernalized state

As advance in development of a plant is not always evidenced by morphological changes, it would be of benefit to be able to detect the qualitative changes which mark the completion of a phase by some microchemical method. A preliminary attempt, using modifications of methods employed by Bassarskaja (**1, 2**) and Richter (**18**), was made to detect completion of the thermo-stage (vernalization) in Marquis wheat seeds.

Marquis wheat was vernalized using 8:3:60 and the seeds were tested, employing the following techniques, after being chilled 4 days and again at completion of vernalization. The control was seed to which 60 per cent water had been added and allowed to germinate for 24 hours at 20° C.

a) Ferric chloride-potassium ferrocyanide method:

1. Sections of embryo cut at a thickness of 75 μ, using a freezing microtome.
2. Sections mounted using a gelatin fixative.
3. Slides treated with 5% ferric chloride for 3 minutes, rinsed thoroughly several times with distilled water, then treated for 3 minutes with 5% potassium ferrocyanide.

Treated in this way the vernalized and unvernalized seeds showed differences in reaction. As the seeds passed toward completion of the thermo-stage (vernalization), the color of the growing point changed from yellow, through green with areas of yellow, to a final dark blue. Tissues other than the growing point stained blue from the start.

b) Change of the iso-electric point:

1. Embryos sectioned and mounted as previously (crushing the embryo tissue on a slide smeared with fixative also proved faster and satisfactory).
2. Slides treated for 15 minutes with McIlwain's buffer solutions of pH 3.6 to 7.0 with intervals of 0.2.
3. Sections stained for 5 minutes in an aqueous solution formed by mixing equal volumes of a 1% solution of eosin and a 1% solution of methylene blue at the moment of staining.
4. After staining, slides rinsed in distilled water and returned to buffer solutions for 6 hours.
5. At conclusion of the 6 hours the pH of the buffers was determined, using a Colemen glass electrode apparatus.

At pH 5.32 sections from unvernalized seeds stained blue. At the end of 4 days of chilling the blue color was obtained at pH 5.13. When vernalization had been completed the embryos stained blue at pH 4.88. Thus, interpreting the results as does GAVRILOVA (**5**), the iso-electric point changed from pH 5.32 to pH 4.88 during vernalization.

Discussion

The development of a plant (advance to reproductive maturity) consists of a continuous series of phases occurring in strict sequence, according to the theory of vernalization or phasic development. The conditions required for the phases differ and the progress of each phase depends on an entire complex of habitat factors. At least four such phases have been reported (**9, 21**). In cereals the first or thermo-phase requires a relatively low temperature for its completion. Light is unimportant during this phase. The second or photo-phase requires a high temperature and a long day.

The aim of vernalization treatment is to induce the young embryo, which has just started growth, to complete the first phase of development during that period when its nutrition is least dependent upon external factors. The progress of the thermo-phase depends on a complex of seed moisture, temperature, and aeration. The experiments with Marquis wheat indicate that within certain limits a higher temperature may compensate for a moisture content below the optimum.

Species and even varieties are specific in their requirements of vernalizing factors (**21**). Hannchen barley responded to 12:3:33 whereas the other cereals did not, the moisture content apparently being too low. Of 125 different treatments Marquis wheat responded most markedly to 8:3:60. Decreases in time to flower of Hannchen barley and of Marquis wheat agree essentially with the results of a number of other workers (**3, 4, 20**). The failure of some investigators (**6, 15, 16, 19**) to obtain acceleration of maturity when cereals were vernalized may possibly be attributed to an ineffective combination of vernalizing factors during treatment.

The majority of investigators obtained increases in yield when spring cereals were vernalized. Reported increases of between 15 and 20 per cent (**3, 4, 15**) were substantiated by my field trials which gave an increase of 21.04 per cent on 5-acre plots.

The findings by FOKEEV and VYROV (**3, 4**) that vernalization resulted in greater increases in yield compared with control grain in areas of drought are not in accord with the results of these experiments.

The results obtained in the experiments involving photoperiodic

responses of Marquis wheat agree with those of McKINNEY and SANDO (**14**), who found that Marquis wheat completes its life cycle quickly with a long photoperiod and a temperature of 70° F. or more throughout its life cycle. These workers also found that Marquis wheat formed seed very sparingly when grown in the greenhouse.

KOSTJUCENKO (**11**) reports that vernalized and unvernalized plants of spring wheat headed 5–9 days earlier in Hibiny (24-hour day) than in Pushkin (18-hour day). Continuous illumination in the experiment on photoperiod response resulted in earlier heading of vernalized plants by 7–19 days and of unvernalized plants by 11–23 days. The acceleration of flowering caused by vernalization was less in continuous light than in normal day length.

The response to vernalization is undoubtedly connected with the type of thermo-phase and the degree to which this phase has been completed prior to seed maturity (**7, 8, 11, 12, 23**), yet the response to treatment is definitely correlated with the after-sowing conditions and place of test. Thus the response to vernalization (shortening of the vegetative period) and length of the phase are not identical, for the after-sowing behavior of plants is governed by the environment, which has a different effect on the development of vernalized and unvernalized plants sown simultaneously. The Marquis wheat grown at various soil temperatures and the vernalized cereals grown simultaneously in the field and greenhouse in 1934 illustrate this point.

While the response obtained was not so simple as that reported by RAZUMOV and SMIRNOVA (**17**) for Bokhara barley grown in the continuous natural sunlight of the Arctic Circle in the greenhouse and in the field, the explanation is probably similar—the effect of temperature on the thermo- and photo-phase of the cereals. The thermo-phase of the unvernalized cereals was retarded at the higher temperatures, while the photo-phase proceeded more slowly at the lower temperatures. The photo-phase of the vernalized plants likewise proceeded more slowly at the lower temperatures than at the high; but because the thermo-phase had been completed during the vernalization treatment the higher temperatures resulted in a rapid completion of the photo-phase, giving an acceleration of 6.5 days in flowering compared with controls at a soil temperature of 38° C.

Not all developmental phases are perceptible morphologically, and not all morphological changes are a direct result of the transition of one phase to another. The morphological manifestations of those qualitative changes which constitute a development phase are subsequent in time to these changes, and the manifestations depend entirely upon the environmental conditions. From this standpoint the significance of the so-called phenological phases in recording the progress of development is practically annulled (**21**). The detection of biochemical changes within the plant, however, may serve to indicate the completion of a particular phase.

The formation of Prussian blue in the tissues of the embryo when treated with ferric chloride and potassium ferrocyanide is due to the reaction

$$4FeCl_3 + 3K_4Fe(CN)_6 = Fe_4[Fe(CN)_6]_3 + 12KCl\,.$$

The absence of Prussian blue in the cells of the growing point of the unvernalized seed may be due to two things: the ferric chloride does not penetrate into the cells or is reduced to ferrous chloride in the tissues (**1, 2**). The latter suggestion is confirmed by the formation of Prussian blue on the addition of potassium ferricyanide to the slides originally staining green. The conclusion is that vernalization produces a change in the oxidation-reduction potential of the cells. The shifting of the iso-electric point upon completion of vernalization indicates that the albumino-lipoids are likewise involved.

All that can be said with any degree of certainty is that some internal readjustments preceding morphological differentiation in the promeristem constitute the essentials of the thermo-phase (**21**). Although supplying little information regarding the exact nature of these changes, the diagnostic experiments suggest it is the physical and chemical properties of the protoplasm which are concerned, and as a matter of course, form the basis for diagnosis.

Summary

1. The reaction of Marquis spring wheat to vernalization was studied through a period of 6 years. Other spring cereals investigated included O.A.C. and Hannchen barley, Banner and Victory

oats, and Prolific rye. Treatment was successful when snow or mechanical refrigeration was used to maintain chilling temperatures. Acceleration of flowering due to vernalization did not exceed 2.3 days when the cereals were grown in the field, with the exception of Hannchen barley which flowered 7 days and ripened 5 days in advance of controls. For plants grown in the greenhouse, the vernalization formula 13:3:33 (13 days chilling at 3° C., the water added being 33 per cent of the original air-dry weight of the seeds) resulted in an acceleration of flowering of 4 days in the case of O.A.C. barley and Prolific rye and of 3 days in the case of Banner oats. Of 125 different combinations of vernalization factors tested, 8:3:60 gave the greatest acceleration of flowering of Marquis wheat when grown in the greenhouse, the greatest average acceleration being 21 days. Increases in yield due to treatment amounted to 21.04 per cent on 5-acre plots. Increase in percentage yield due to treatment was less in time of drought than when growing conditions were favorable. Air drying vernalized seed reduced the percentage germination by 9.5.

2. Acceleration of flowering of Marquis wheat, O.A.C. barley, Banner oats, and Prolific rye due to vernalization was less when grown under continuous illumination than in normal day length, compared with controls receiving the same photoperiod. Increasing the photoperiod from normal day length to 24 hours shortened the vegetative period of untreated plants by 11–23 days and that of vernalized plants by 7–19 days.

3. Marquis wheat was grown at soil temperatures ranging from 22° to 44° C. Behavior showed earlier flowering of both vernalized and control plants as the soil temperature rose from 22° to 34° C., and a greater acceleration of flowering or a larger number of flowerings, due to vernalization, in plants grown at higher soil temperatures. While the dry weights of control and vernalized plants decreased as the soil temperature rose from 22° to 44° C., the top/root ratio rose to a maximum at 34°, falling to approximately the original value as the soil temperatures rose above 34° C. The growth of vernalized Marquis wheat in the greenhouse, with its higher temperatures, likewise resulted in a greater acceleration of flowering than when grown in the field.

4. Two microchemical methods were used to diagnose the vernalized state of Marquis wheat. Treatment of embryos with ferric chloride and potassium ferrocyanide showed a change in the ability of the growing point to reduce ferric chloride as vernalization progressed. A shifting of the iso-electric point of the embryo tissues from pH 5.32 to pH 4.88 upon completion of vernalization was detected by the use of McIlwain's buffers and staining with eosin and methylene blue.

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CHROMOSOME NUMBER IN SOME TULIP HYBRIDS[1]

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JOHN M. BELLOWS, JR.

Introduction

During some studies on interspecific and intervarietal hybridization in the genus *Tulipa*, it developed that certain triploid varieties and species as well as a pentaploid species could be used as parents in the formation of hybrids. These hybrids are interesting in that the majority of them are aneuploids. HALL (**7**) has recently reported similar results with the same types of crosses, although he does not list the parents involved. He also points out that such aneuploid plants have not been previously reported either in species or in garden varieties, although *T. galatica* ($2n=32$) might be placed in such a category (**13, 23**). However, HALL (**7**), reporting on some unpublished work of DARLINGTON and UPCOTT, considers the extra chromosomes in this form as supernumerary, and adds that the chromosome number is variable.

The successful hybrids involving either triploid or pentaploid parents are as follows:

T. lanata Regel. ($2n=36$)×*T. tubergeniana* Hoog. ($2n=24$)
Inglescombe Yellow ($2n=36$)×John Ruskin ($2n=24$)
John Ruskin ($2n=24$)×Inglescombe Yellow ($2n=36$)
Clara Butt ($2n=24$)×Inglescombe Yellow ($2n=36$)
T. clusiana D.C. ($2n=60$)×*T. chrysantha* Boiss. ($2n=48$)
T. chrysantha ($2n=48$)×*T. clusiana* ($2n=60$)
T. clusiana ($2n=60$)×*T. stellata* Hook. ($2n=24$)
T. clusiana ($2n=60$)×self

The chromosome numbers of the parents involved in these crosses have been reported previously (**13, 14, 23, 7, 25**). It is the purpose of this paper to report the chromosome numbers of such hybrids, emphasizing especially the contribution of the triploid or pentaploid parent.

[1] Contribution no. 494, Maryland Agricultural Experiment Station.

Material and methods

All chromosome determinations were made from root tips or droppers. These tips were removed from seedlings or one- or two-year-old bulbs, which were produced as the result of crosses made in 1934 and 1935. Fixation was made in Allen and Wilson's modification of Bouin's fluid, and the material was then treated with the usual xylol-paraffin technique. Sections were cut 18–22 μ thick and stained in iron-alum haematoxylin. Observations were made with the aid of a 15× compensating ocular and a 90× 1.3 apochromatic oil immersion objective.

In each of the following crosses, the chromosome number contributed by the triploid or pentaploid has been derived by subtracting the gametic number of the other parent from the chromosome number obtained in the root tips of the hybrid seedlings. Information from other crosses has demonstrated that the diploids and tetraploids produce the expected chromosome number in the gametes.

Results

Hybrids with triploid parent

1. *T. lanata* (3*n*)×*tubergeniana* (2*n*)

In the material collected from the plants of this cross it was possible to determine the chromosome number of 35 hybrids. The results (table 1) indicated that the triploid parent contributed 15 and 18 chromosomes to the hybrids most often, although the variation in the functional gametes may extend from 12 to 21.

TABLE 1

Chromosomes from triploid..	12	13	14	15	16	17	18	19	20	21	22
No. of plants.....	1	2	4	10	2	3	8	0	2	3	0

2. Inglescombe Yellow (3*n*)×John Ruskin (2*n*)

This cross is similar, in so far as chromosome number of the parents is concerned, to the previous one. The results are somewhat comparable in that the triploid contributed 15 and 18 chromosomes most frequently, although those gametes with 13 chromosomes were effective. The variation was from 12 to 20 chromosomes in the 28 hybrids obtained (table 2).

TABLE 2

Chromosomes from triploid	12	13	14	15	16	17	18	19	20
No. of plants	2	6	2	8	2	1	6	0	1

3. John Ruskin (*2n*)×Inglescombe Yellow (*3n*)

When the triploid is used as the male parent of the hybrid, the results (table 3) are considerably different. Although the range of chromosome numbers is about as extensive, the hybrids with 25 chromosomes are predominant in the progeny, showing that the triploid contributed 13 chromosomes to the hybrids more often than any other number.

TABLE 3

Chromosomes from triploid	12	13	13+	14	15	16	17	18
No. of plants	2	19	1*	6	1	6	2	1

*Thirteen plus a fragment.

4. Clara Butt (*2n*)×Inglescombe Yellow (*3n*)

From this cross only a few plants were obtained and the chromosome numbers of three of these were determined. One hybrid had 25 chromosomes, one had 25 plus a fragment, and the third had 26 chromosomes.

Hybrids with pentaploid parent

It is apparent that the progeny are few in those crosses in which one parent was a pentaploid. Such hybrids are difficult to obtain, and furthermore the few seeds are either non-viable or the seedling succumbs so that a bulblet never forms. The following results, however, indicate that it is possible to obtain viable seed from crosses involving the pentaploid as one parent and a diploid or tetraploid as the other. It was also possible to obtain progeny from a selfed pentaploid.

5. *T. clusiana* (*5n*)×*chrysantha* (*4n*)

Although only three seedlings from which chromosome counts could be obtained were available, the variation in the functional gametes of the pentaploid is apparent. In one the pentaploid contributed 21 chromosomes, in another 22, and in the third 29.

6. *T. chrysantha* (*4n*)×*clusiana* (*5n*)

The results from the reciprocal of this cross (table 4) were determined from seven seedlings which survived. The hybrids ranged in

chromosome number from 49, just one more than the somatic number of the tetraploid, to 60, the pentaploid number. The pentaploid contributed 26 and 25 chromosomes respectively to the hybrids the greater number of times.

TABLE 4

Chromosomes from pentaploid	25	26	27	28	29	30	31	32	33	34	35	36
No. of plants	2	3	0	0	0	1	0	0	0	0	0	1

7. *T. clusiana* (5*n*)×*stellata* (2*n*)

Seven seedlings from this pentaploid-diploid cross showed the usual variation in their chromosome number (table 5). There is a slight predominance of 37 and 40 chromosome hybrids, indicating that the pentaploid as the female parent contributed 25 and 28 chromosomes respectively the greater number of times. However, the chromosome numbers of all the progeny range from 22 to 28.

TABLE 5

Chromosomes from pentaploid	22	23	24	25	26	27	28
No. of plants	1	0	1	2	1	0	2

8. *T. clusiana* (5*n*)×self

Since there is no means of determining how many chromosomes the male and the female each contribute to the hybrid, table 6 is presented to show the chromosome numbers of the progeny.

TABLE 6

Chromosome number of hybrids	47	48	49	50	51	52	53	54	55	56	57	58	59	60
No. of plants	1	2	0	1	0	0	1	1	0	0	1	0	0	1

Discussion

A great many studies concerning meiosis in triploids and pentaploids have indicated that the reason for sterility in these types was due to the irregular gamete formation. Moreover it has been assumed that the chromosome distribution during the divisions showed what the gametes would have.

Practically equal division of chromosomes to the gametes, or the number representing one-half the somatic number, has been found by the investigators in the following genera: *Impatiens* (19), *Zea*

(11), *Lycopersicum* (9), *Petunia* (20), *Oenothera* (3), *Triticum* (22), *Hyacinthus* (1), *Datura* (2), and *Lilium* (18).

NEWTON and DARLINGTON (13), working with triploid and pentaploid *Tulipa*, and CHANDLER, PORTERFIELD, and STOUT (4) in a recent paper on the meiosis of triploid *Lilium*, found numerous irregularities in the prophase of the first division, so that lagging and eventual elimination of chromosomes often occurred. As a result, gametes would be formed which contained a smaller number of chromosomes than if this elimination had not taken place.

Recently SATINA and BLAKESLEE (15, 16) examined meiotic divisions of both male and female gametophytes in triploid *Datura* and found practically equal chromosome distribution in the first division, but subsequent irregularities reduced the population of these gametes and increased the population with unevenly divided or lower chromosome numbers. WOODS (24) in *Tulipa* observed the nearly equal distribution of chromosomes in the gametes at the first division, but some irregularities were present in later divisions so that the resulting gametes contained aneuploid numbers.

DARLINGTON (5) has stated: "The zygote progeny (however) do not correspond at all with the gametic proportions." The results of this immediate study indicate that the triploid, serving either as the male or female parent, rarely contributes the 18 chromosomes expected as a result of equal chromosome-carrying gametes. On the contrary, it is obvious that the functional gametes of the triploid carry considerably less than 18 chromosomes. When used as a female parent, in three different crosses, it contributed 15 chromosomes most frequently. As the male parent, 13 and 14 were the predominating number of the functional gametes.

This behavior of the triploid gametes of *Tulipa* is not unusual in the light of similar work now in the literature. There is evidently an elimination of those gametes carrying the higher chromosome number, so that the functional gametes contain numbers equaling or closely approximating the diploid number. As WOODS (24) has pointed out in a previous paper, bridging and fragmentation are common in the meiotic division of a triploid *Tulipa*. This probably accounts in part for the eventual elimination of chromosomes in the gametes of these triploids.

HALL (7) mentions similar results in some triploid *Tulipa*. He obtained aneuploid seedlings where the triploid has contributed from 12 to 22 chromosomes. He does not discuss the parents nor the way in which the cross was made.

DERMEN (6) crossed diploid *Malus* on triploid and obtained aneuploid hybrids ranging from the diploid to the triploid chromosome number. LEVAN (10), working with *Allium*, made reciprocal crosses between diploids and triploids and found that "the elimination or non-formation of aneuploid zygotes was greater when the embryo developed on a diploid than on a triploid." When the diploid served as the female parent, 53 of the 71 hybrids had the diploid number. When the triploid served as the female parent, aneuploid hybrids ranging from the diploid to the triploid number were obtained. MCCLINTOCK (12), working with *Zea*, found that in the triploid there was a "decided selection against extra chromosome-carrying male gametes and a less obvious selection against extra chromosome-carrying eggs." When the triploid served as seed parent, this cross indicated that not only gametes with the diploid number (no extra chromosomes) were functional, but also those with one and two extra chromosomes in excess of the diploid number. STOUT (21) made reciprocal crosses between diploid and triploid species of *Hemerocallis* and found that the triploid contributed the same number as the diploid most frequently to the hybrids, but the aneuploid numbers ranging up to twice this also functioned.

SATINA, BLAKESLEE, and AVERY (17) studied the chromosome number in the seed obtained from a triploid×diploid cross and found that there were 816 times the number of diploid individuals expected on the basis of random assortment of chromosomes. LAMMERTS (8), in a diploid×triploid cross of *Nicotiana*, found that the triploid gametes with the expected chromosome number, assuming equal distribution of chromosomes in the gametes, functioned most frequently.

According to HALL (7), "the pentaploid *T. clusiana*, though grown in quantity for commerce, is generally regarded as sterile, as might be expected from its pentaploid constitution." He states, however, that seedlings have been obtained on one or two occasions, but the parentage is unknown and occasionally this species set seed spontaneously. The seedlings had the pentaploid chromosome num-

ber, while those which appeared spontaneously were aneuploids with 50–52 chromosomes.

In the crosses involving *T. clusiana*, the pentaploid, variations in the hybrid seedlings are indicative of the irregular gamete formation in pentaploids. NEWTON and DARLINGTON (**14**) pointed out that this species forms a large proportion of aneuploid gametes. From observations of the first meiotic division of the pollen mother cells, they found associations at metaphase including quinquevalents, quadrivalents, trivalents, bivalents, and as many as ten univalents. The random distribution of the 60 chromosomes to the respective daughter cells would necessarily result in irregular numbers in many of the gametes, and confirmation of this is found in the results of the four *clusiana* crosses.

In the first of these, *T. clusiana*×*chrysantha*, assuming that the tetraploid male gametes form normal gametes of 24 chromosomes as in preliminary reciprocal crosses with a diploid, the pentaploid has formed functional gametes of 21, 22, and 29 chromosomes. In the second cross, *T. chrysantha*×*clusiana*, the pentaploid pollen showed great variation in chromosome number, for the range in the functional gametes extended from 25 to 36, although the frequency is highest in the neighborhood of 25 and 26.

The *T. clusiana*×*stellata* cross showed less variation in the chromosome number of the functional gametes, the range extending from 22 to 28, and no outstanding high frequency for any particular one of these.

The most unusual fact concerning the last of these crosses, *T. clusiana*×self, is that it was actually possible to obtain viable seed that survived. The ranges in the chromosome numbers of the functional gametes are hardly enlightening, except to show that there is great variation. Obviously there are no means of determining how many chromosomes the male or the female contributed.

From a consideration of these four crosses involving the pentaploid *T. clusiana*, it will be seen, as in the case of the triploid, that the pentaploid might form gametes containing one-half the somatic chromosome number, but they do not function as often as those gametes containing less than the expected number. From the work of NEWTON and DARLINGTON (**14**) previously mentioned, however,

one does not anticipate the formation of such equal chromosome-bearing gametes. Rather, as in the triploid, there has probably been an elimination of chromosomes so that the functional gametes carry less than the expected number.

Summary

1. Seedlings have been obtained from reciprocal crosses involving both triploid and pentaploid forms of *Tulipa*. The other parents of these crosses were diploids and tetraploids.

2. The chromosome numbers of these progeny have been determined. The majority of them are aneuploids.

3. The triploid, either as a male or female parent, tends to contribute less than one-half the somatic number of chromosomes. This is probably explained by the irregularities in the meiotic divisions of this type.

4. Although the number of progeny from the pentaploid is small, a similar condition exists.

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FURTHER EXPERIMENTS ON THE RELATION OF VITAMIN B_1 TO THE GROWTH OF GREEN PLANTS[1]

JAMES BONNER AND JESSE GREENE

(WITH ONE FIGURE)

Introduction

In an earlier paper it has been shown (**5**) that some plants grown in sand culture in the greenhouse may respond with marked increases of growth to the addition of minute amounts of vitamin B_1 in the nutrient solution. The present paper reports further data relative to the vitamin B_1 economy of the green plant.

MATERIAL AND METHODS.—Plants were grown in quartz sand washed free of organic matter and contained in 2-gallon glazed crocks supplied with drainage. Shive's R_2S_5 nutrient containing the minor elements was used for the majority of the experiments. The nutrient solution was made up from chemically pure reagents in order to avoid contamination by organic matter as far as possible. The nutrient was supplied at the rate of 250–500 cc. per crock on alternate days, the amount depending on the size of the plants and on weather conditions. On intervening days the crocks were supplied with tap water. In experiments where vitamin B_1 was to be used, it was added at the rate of 0.01 mg. per liter of nutrient solution unless otherwise noted.

A number of the experiments were conducted during the winter of 1938–1939. The light conditions prevailing during this time permitted vigorous growth in the greenhouse.

The "long photoperiod" conditions referred to here consisted of daily light periods varying from 18 to 20 hours. The natural day was supplemented with light from Mazda lamps which gave an intensity of approximately 75 foot-candles at the leaf surface. Conditions of "short photoperiod" refer to a daily light period of 9 hours.

[1] Work carried out with the aid of the Works Progress Administration, Official Project no. 665-07-3-83, Work Project L-9809.

Vitamin B_1 determinations were carried out by means of the *Phycomyces* assay (**8, 4**). Samples of the leaves to be analyzed were collected, generally between 11 A.M. and 2 P.M., dried at 60° C. for 48 hours, ground, and the vitamin determined in triplicate 10-mg. samples. The values obtained with the assay in this manner were found to be in good agreement with those obtained on the same material by the thiachrome fluorescence method after extraction, adsorption on Fuller's earth, and elution of the vitamin.[2] Because of its relative ease and simplicity, the *Phycomyces* assay was used in preference to the fluorescence method. It seems unlikely that substances normally present in leaf tissue interfere in any way with the *Phycomyces* assay, since added vitamin can be recovered quantitatively in the presence of such tissue (table 1).

TABLE 1

PHYCOMYCES ASSAY FOR VITAMIN B_1 IN THE PRESENCE OF LEAF TISSUE

CONTENTS OF TEST FLASKS (IN ADDITION TO 10 CC. PHYCOMYCES NUTRIENT)	VITAMIN B_1 (GAMMA)	
Nothing	0.00	
0.10 gamma vitamin B_1	0.10	
10 mg. ground leaf tissue (Schinus molle)	0.048	
10 mg. ground leaf tissue + 0.10 γ vitamin B_1	0.148	
	0.100	100% recovered
10 mg. ground leaf tissue (Eucalyptus)	0.054	
10 mg. ground leaf tissue + 0.10 γ vitamin B_1	0.156	
	0.102	102% recovered

Investigation

INFLUENCE OF VITAMIN B_1 ON ACCUMULATION OF DRY WEIGHT

The rate of accumulation of dry weight, both of root and of shoot, appears to be increased under the influence of added vitamin B_1 (table 2); and with certain species such as mustard, this increase may amount to several hundred per cent. Results similar to those presented in table 2 have been obtained with numerous other species of plants, for example *Agrostis tenuis* and *Poa trivialis*. These were

[2] We are indebted to ERICK HEEGARD for the thiachrome determinations, and to E. WADDELL for the execution of the *Phycomyces* assays.

grown in sand under conditions similar to those just outlined and the grass was clipped periodically. In 2 months the cultures of *P. trivialis* which received vitamin B_1 produced a total of more than 13 gm. dry weight of clippings per crock whereas the control crocks produced but 2.05 gm. *Agrostis tenuis* which received vitamin B_1 produced during the same time more than four times as great a dry weight of clippings as the controls.

Plants grown in soil may be induced to accelerate their rate of dry weight deposition by additions of vitamin B_1, as shown by the following experiment. *Cosmos* seedlings were planted in 4-inch pots in an open garden soil and maintained in the greenhouse. The experimental plants were watered with a solution containing 0.1 mg. of vitamin B_1 per liter while the controls received tap water. After 6 weeks the plants supplied with the vitamin were found to have an average dry weight per shoot (stem and leaves) of 0.33 gm. while the control plants had an average of 0.080 gm. per shoot, or less than one-fourth that of the treated plants.

TABLE 2

RELATION OF ADDED VITAMIN B_1 TO ACCUMULATION OF PLANT DRY WEIGHT

Species	Length of experiment (weeks)	No. plants	Dry weight (gm. per plant)					
			Tops		Percentage increase	Roots		Percentage increase
			Control	B_1		Control	B_1	
Xanthium pennsylvanicum (short photoperiod)	7	80	0.72	1.15	160	0.061	0.12	197
Brassica alba (short photoperiod)	6	40	0.064	0.29	453	.....	.....	.....
Brassica nigra (short photoperiod)	6	40	0.047	0.175	372	.....	.....	.....
Cosmos (long photoperiod)	8	50	0.0675	0.110	163	0.0134	0.0183	137

In all these cases, as well as in many others, the plants which received the vitamin exhibited a greater general vigor than the control plants, as indicated by taller shoots, larger leaves, and more extensive root system. On the basis of the results presented in table 2,

it seems justifiable to conclude that this increase in vegetative vigor is accompanied by an actual increase in accumulation of dry matter.

Effective concentrations of vitamin B_1

Table 3 gives the results of an experiment in which *Cosmos* seedlings, grown in sand culture, were supplied with nutrient solution containing varying amounts of vitamin B_1. The plants were

TABLE 3

Relation of vitamin B_1 concentration of nutrient solution to dry weight deposition by Cosmos seedlings. Plants maintained under conditions of long photoperiod

Concentration of nutrient solution	Dry weight (gm. per shoot)	Percentage increase over control
None (nutrient only)	0.058	.....
Mg. per liter		
0.1	0.105	81
0.01	0.138	138
0.001	0.111	91
0.0001	0.075	29

harvested 4 weeks after the appearance of the first leaves, and dry weights determined. The optimum concentration of vitamin B_1 for the promotion of dry weight deposition appears to be 0.01 mg. per liter of nutrient solution under these conditions, but a detectable effect was exerted by nutrient solution containing only 0.0001 mg. or 0.1γ of vitamin B_1 per liter.

Effects of vitamin B_1 over extended periods

In an earlier paper (5), as well as in the experiments of table 2, the plants were harvested and the results obtained after periods of 4–10 weeks. In figure 1 are presented the growth curves, as measured by total height of shoot, of two sets of *Ceratonia siliqua* (carob tree) seedlings maintained in sand culture for 1 year. Although the control plants grew vigorously, the plants which received vitamin B_1 grew still more vigorously throughout the experimental period. After 1 year, the plants which received the vitamin continued to

grow at a faster rate than the controls. Similar although less striking results were obtained with seedlings of *Prunus ilicifolia* which were grown in soil in the open during a period of 1 year. Growth measurements made in experiments which extended through several months

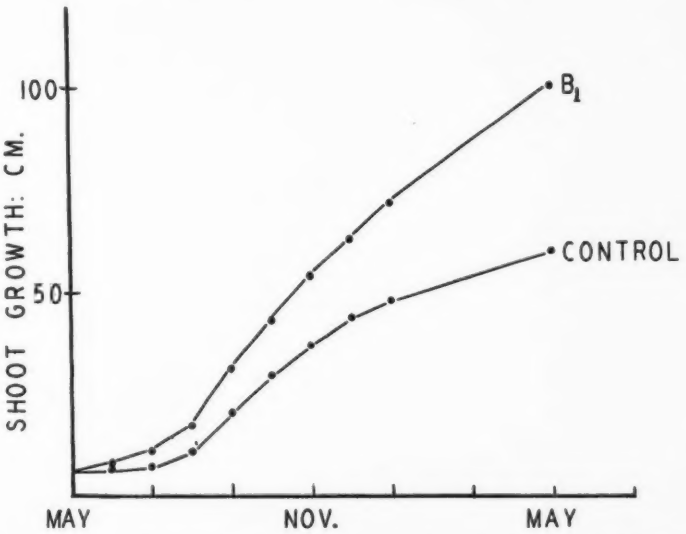

FIG. 1.—Growth of seedlings of *Ceratonia siliqua* in sand culture with and without addition of vitamin B_1 (0.01 mg. per liter).

are given in table 4. In every case the treated plants continued to grow more rapidly than the controls throughout the course of the experiment. Additions of vitamin B_1 apparently bring about a lasting rather than a temporary promotive effect on plant growth.

RELATION OF VITAMIN B_1 CONTENT TO PLANT RESPONSE

It has been pointed out (5) that certain species do not respond with increased growth to additions of the vitamin. Among such species are corn, wheat, pea, garden bean, and tomato. It is known both for pea (1, 3) and for tomato (7) that vitamin B_1 is indispensable for the growth of isolated roots, thus presumably it is indispensable for root growth in intact plants of these species.

In table 5 are tabulated vitamin B_1 contents of the leaves of several species of plants.[3] These leaves were taken from the control plants of experiments in which the growth response to added vitamin was also determined. The species found to respond to additions of the vitamin with increased growth possessed leaf vitamin contents between 0.0 and 6.0 mg. per kg. dry weight of leaves. The vitamin content of tomato leaves was found to be 18 mg. per kg., or at least three times as great as that of the species responsive to vitamin B_1.

TABLE 4

RESPONSE OF SEEDLINGS TO ADDED VITAMIN B_1

SPECIES	LENGTH OF EXPERIMENT	NO. PLANTS	GROWTH MEDIUM	GIVEN B_1	NEW SHOOT GROWTH AT END OF EXPERIMENT (CM.)		RATIO OF B_1/CONTROL (%)
					B_1	CONTROL	
Ceratonia siliqua....	1 year	8	Sand	Alternate days	93	54	172
Myrsine africana....	5 months	24	Sand	Alternate days	34	20	170
Daphne odora.....	6 months	8	Sand	Alternate days	3.6	1.1	327
Correa ventricosa...	6 months	8	Sand	Alternate days	36	32	112
Camellia japonica...	6 months	16	Sand	Alternate days	23	6.4	360
Prunus ilicifolia.....	1 year	12	Soil*	Weekly	233	178	131
Cedrus libani.....	5 months	16	Soil*	Weekly	42	17	247

* Grown outdoors.

The leaves of pea plants contain two or more times as much vitamin as the leaves of responsive species.

It has been demonstrated in the case of the pea plant that vitamin B_1 is formed in the green leaves under the influence of light (**5**), and that in the normal plant the vitamin B_1 required by the root is supplied presumably by these leaves. Species with relatively high contents of leaf vitamin B_1 may not be limited in their growth by the available amounts of this substance. Those species of table 5 with relatively low (less than 6.0 mg. per kg.) content of leaf vitamin

[3] The determinations summarized in table 5 represent the vitamin contents of the leaves of the individual species under the conditions of the particular experiment. It has been found, as would be expected, that the vitamin content of leaves varies with external factors. This will be the subject of a later report.

appear to be limited in their growth by the available vitamin B_1. The response of these latter species to added vitamin B_1 is not completely correlated with leaf vitamin content. Thus *Poa trivialis* with 4.2 mg. per kg. dry weight of leaves responds more strikingly than does *Agrostis tenuis* with 6.0 mg. of vitamin per kg. dry weight of leaves. On the other hand, *Brassica alba* responds to added vitamin somewhat more than does *B. nigra*, although the leaf vitamin content of the former is higher than that of the latter. That leaf vitamin B_1 content should not be completely correlated with response to

TABLE 5

B_1 CONTENT OF LEAVES OF VARIOUS SPECIES OF PLANTS GROWN IN SAND CULTURE

PLANT	CONDITIONS	B_1 CONTENT OF LEAVES MG./KG. DRY WEIGHT	RESPONSE TO ADDED VITAMIN
Tomato		18.0	None
Pea		13.0	None
Brassica alba	Short photoperiod	4.9	+
Brassica nigra	Short photoperiod	3.0	+
Agrostis tenuis		6.0	+
Poa trivialis		4.2	+
Cosmos (autumn)	Long photoperiod	5.0	+
Xanthium pennsylvanicum	Short photoperiod	3.4	+
Ceratonia siliqua		3.0	+
Cattleya (hybrid)		1.8	+
Camellia japonica		0.0	+

added vitamin would be in accordance with the view that although available vitamin B_1 may be a factor limiting the growth of these species, there may be still other growth factors which are to different degrees also limiting in the several cases. Leaves of *Camellia japonica* were found to contain no detectable amount of the vitamin. When vitamin B_1 was added to the nutrient solution, plants of this species were found to thrive in sand culture and to approximate in luxuriance plants grown in rich soil. Control plants which received no vitamin were found to grow very poorly in sand culture. It would seem possible that *Camellia* plants depend for their supply of vitamin B_1 largely on the rich soil with which they are supplied in usual horticultural practice.

Relation of vitamin B_1 nutrition to vitamin content of the leaf

When vitamin B_1 is supplied to the roots of plants grown in sand culture there is an increase in the amount of vitamin present in the leaves of these plants (table 6). It has not yet been possible to decide

TABLE 6

Accumulation of vitamin B_1 in leaves of plants grown in sand culture with added vitamin

Species	Vitamin content of leaves (mg./kg. dry weight)	
	Supplied B_1	Control
Brassica alba (long photoperiod)	15.8	6.0
Brassica nigra (long photoperiod)	6.4	3.9
Agrostis tenuis	8.6	5.8
Poa trivialis	7.2	4.4
Cosmos (long photoperiod)	6.0	5.0

whether this increase is to be attributed solely to passive uptake of the vitamin or to an increased synthesis of the vitamin by the treated plants.

Discussion

Although the addition of vitamin B_1 exerts a promotive effect on the vegetative growth of many species of green plants, it has not yet been found to exert any specific effect on the initiation of the flowering state (**6**). In the present experiments, and also in others not reported here, plants have been maintained in culture until the flowering state was attained. In no case was an effect on the time of flowering or on the number or size of flowers or fruits observed, other than such as might be expected from the greater vegetative vigor of the treated plants. In no case have additions of vitamin B_1 been found to be in any way detrimental to flower production in the present experiments.

It has been shown (**2**) that the growth response of etiolated ex-

cised pea embryos of different strains to added ascorbic acid is a direct function of the quantity of the acid which each particular strain is capable of synthesizing for itself. It would appear that the response of different species of green plants to additions of vitamin B_1 similarly depends on the amount of this growth factor which each species is able to synthesize under the prevailing environmental conditions.

Summary

1. Species of plants grown in sand culture under greenhouse conditions respond to addition of vitamin B_1 to the nutrient solution (0.01 mg. per liter) with marked increases in rate of dry weight accumulation. A similar influence of added vitamin on dry weight accumulation of plants grown in soil is also demonstrated.

2. Carob trees supplied on alternate days with vitamin B_1 in sand culture during 1 year continued to exhibit a greater growth rate than controls which did not receive the vitamin. This and other experiments indicate that the promotive effect of added vitamin is lasting rather than temporary.

3. Tomato, which does not respond to addition of vitamin B_1 with added growth, was found to contain at least three times as much vitamin in its leaves as was found in the leaves of other plants (*Cosmos*, *Poa*, *Brassica*, etc.) which do respond to such addition with added growth. *Pisum sativum*, another species unresponsive to additions of the vitamin, was found to contain more than twice as high a concentration of leaf vitamin as the responsive species. It is suggested that the amount of vitamin B_1 synthesized by the leaves of a given species regulates, at least in part, the response of that species to additions of the vitamin. It is possible that leaf vitamin content might be used to diagnose whether or not addition of vitamin B_1 would promote the growth of a given crop or species of plant.

4. When vitamin B_1 is supplied to the roots of plants grown in sand culture, the vitamin content of the leaves is increased over that of control plants not supplied with the vitamin.

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COLOR AND KEEPING QUALITIES OF CUT FLOWERS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 508

MARSHALL S. NEFF

Roses, such as the Hollywood variety, as well as many other red or pink flowers, take on a faint bluish cast under some storage conditions. Similarly flowers which have been held in storage at times do not last as long at ordinary room temperatures as do fresh flowers. Preliminary experiments indicate that these difficulties may be obviated by means readily available to those who are concerned with the production, storage, and marketing of cut flowers.

In the first experiment freshly cut Hollywood rosebuds were stored at 35°–40° F. in a 70-liter metal container while in a refrigerated chamber. Water to a depth of $\frac{1}{4}$ inch in the bottom of the container and a sheet of window glass over the top, in place of the metal lid, maintained a high humidity within the container. The rosebuds were divided into three groups previous to being placed in the container. In group 1 the lower portions of the stems were immersed in a flask containing a commercially available solution designed to maintain flowers in a fresh condition over a considerable length of time provided the flower stems remain in it. Two and one-half times the amount of sugar recommended by the manufacturers was added to the solution. In group 2 the flower stems were kept dry, an empty flask being placed over their ends to keep the stems out of the water which covered the bottom of the container. In group 3 the lower portions of the stems were placed in the commercial solution (with the added sugar as noted) for an hour; they were then removed from the liquid and the bases of the stems covered with a dry empty flask as in group 2, and also placed in the container. All lots were illuminated, as soon as the groups were in place, with light from a 160 watt Mazda lamp set in a small reflecter and suspended 8 inches above the glass of the metal container in the cold room. The light was first passed through a filter consisting of water to which had been added sufficient copper sulphate to produce a

light blue color. The flowers received approximately 50 foot-candles of light over a 168-hour period. During the last 17 hours of storage the copper sulphate solution was removed and the light intensity reduced so that the flowers received approximately 13 foot-candles. At the end of the storage period the color of the flowers was still fresh and lively, perhaps slightly less intense than in fresh flowers, but there was no bluing tendency. The flowers were removed from

TABLE 1

EFFECT OF STORAGE CONDITIONS UPON SUBSEQUENT KEEPING QUALITIES OF HOLLYWOOD ROSES AT ROOM TEMPERATURE; STORAGE PERIOD 185 HOURS AT 35°–40° F.

GROUP	HUMIDITY	HOURS WITH STEMS IN SOLUTION*	HOURS STORED DRY	APPROXIMATELY 50 FOOT-CANDLES (FILTERED)†	APPROXIMATELY 13 FOOT-CANDLES (UNFILTERED)	NUMBER OF TURGID FLOWERS AT ROOM TEMPERATURE. FOUR FLOWERS USED IN EACH GROUP. STEMS IN WATER					
						1 DAY	4 DAYS	5 DAYS	6 DAYS	8 DAYS	9 DAYS
1	High	185	0	168	17	4	4	4	4	1	1
2	High	0	185	168	17	4	4	4	1	1	.....
3	High	1	184	168	17	4	4	2	.....	.....	.....
4 Fresh roses (check)	.....	.....	.....	.....	.....	4	4	1	.....	.....	.....

* A commercial product mixed with water and sugar.

† Light filtered through a layer of water to which was added sufficient copper sulphate to produce a light blue color.

storage and taken to the laboratory where the temperature was approximately 70° F. They were placed with the lower portions of their stems in water in order to compare their keeping qualities with freshly cut buds also held at room temperature. The flowers stored dry tended to droop during the storage period, but they revived at room temperature when their stems were in water (table 1).

The table indicates that roses which have been stored may last longer in water at room temperature than fresh roses under the same conditions. The one-hour period of immersion of the stems in the commercial solution and subsequent dry storage proved less effective in maintaining good color and a fresh condition than when the stems were not placed in the commercial solution first. On the

other hand, maintenance of the stems in the solution during the entire storage period gave the best results as to color and keeping qualities. By the sixth day at room temperature the fresh roses (group 4) were a dull bluish color and the petals were wilted and dried. Treated roses (group 1) were turgid and retained a lively pink color. The roses of group 2 retained a brighter color and remained fresh longer than fresh flowers which had not been stored.

In a second experiment roses were held one week in a sealed metal container under conditions of 34°–38° F., high humidity, darkness, and the stems out of water or solution of any kind. During the week in darkness the petals lost the characteristic glow of fresh flowers and there was a tendency to become blue. At the end of the week the flowers were removed from the container but kept in the cold room at the same temperature as previously. The stems of these flowers were then placed in a solution of the following composition: 1000 cc. water; 100 gm. glucose or sucrose; 0.5 gm. potassium nitrate. With the bases of the stems in this solution they were then returned to the container. Window glass was again substituted for the metal cover so that it was possible to expose the flowers for 185 hours to the same conditions of light, temperature, and humidity as detailed in the first experiment. During this treatment the bluish color which developed during the storage period in darkness was eliminated. The roses remained turgid after they were removed to room temperature and the stems placed in water. They retained a lively color for a considerable time. No comparison was made with fresh flowers in this case.

The commercial solution used in the first experiment was also tested in conjunction with low temperature, low humidity, and direct Mazda light. Freshly cut rosebuds were placed in the 34°–38° F. room with the lower portions of their stems in a jar containing the commercial solution. A beaker full of dry calcium chloride was placed next to the flowers and a bell jar inverted over both the flowers and the calcium chloride. A short piece of glass tubing running through a rubber stopper in the top of the bell jar permitted inward and outward diffusion of gases. The exact humidity maintained was not determined but the lack of any accumulation of water droplets on the sides of the bell jar indicated a much lower humidity than previously used. No light filter was used. The flowers received direct Mazda light for 185 hours. When removed from the ball jar

to an open room at 70° F. and the stems placed in water the treated flowers had better keeping qualities than fresh roses. In addition, the color was very similar to that of group 1 in the first experiment. In this latter experiment the foliage was bleached somewhat and tended to wither and dry more rapidly than in the untreated specimens.

In another experiment freshly cut rosebuds were divided into two lots. The stems of one group were placed in the potassium nitrate-sugar solution, already mentioned, and the stems of another in tap water. No metal container or bell jar was used. The flowers were placed in a room at 70° F. Suspended in the room was an arc light, screened in with glass to eliminate any injurious ultra-violet rays. Approximately 800 foot-candles of light reached the flowers. They were maintained in the light for about 18 hours. At the end of this period the color was still bright and lively but the intensity was decreased slightly. When these treated flowers were removed to an open room at 70° F. and their stems placed in water under usual conditions, the color of those which had the lower portions of the stems in the potassium nitrate-sugar solution during the 18 hours of light treatment remained lively and fresh until the petals wilted. They showed much less tendency to turn blue than did untreated roses. Roses that had been in water only, during the light treatment, had less tendency to turn blue than those without any light treatment, but their petals began to take on a bluish cast with maturity. The treated roses of either type lasted longer than fresh flowers when held at room temperature.

Further investigations are now in progress to determine the optimum conditions for maintaining color and keeping qualities of flowers. It is obvious that light, either from a screened arc lamp, direct from a Mazda lamp, or filtered through a copper sulphate solution, is of great aid in extending the life of cut flowers in water at room temperature and preventing the tendency of red flowers to turn blue. This is particularly true when used in association with dry storage, or when the stems are immersed in appropriate solutions.

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LAYERING IN EASTERN WHITE PINE

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(WITH ONE FIGURE)

Natural layering appears to be exceedingly rare in *Pinus*. MAYR (2) stated that it occurs in the group but did not mention specific examples. In his discussion of reproduction by layering among conifers, COOPER (1) cited MAYR and also mentioned a possible example of layering in *Pinus montana* described by SCHRÖTER. Apparently natural layering in *Pinus* has not previously been reported in America. The example here reported was observed in the Yale Demonstration and Research Forest, near Keene, New Hampshire, during the summer of 1938.

The parent tree, a white pine, originated about 30 years ago at the foot of a short slope in an old field. The soil was Merrimac coarse sand. Ground vegetation was sparse and the pine trees were scattered so that considerable erosion occurred on the slope. Eroded material accumulated to a depth of 15–25 cm. around the tree, covering portions of the lowest whorl of branches with a mixture of mineral soil material and organic debris. One of the basal branches on the south side of the tree developed roots. The parent tree and the rooted basal branch, which had assumed an upright habit, are illustrated in figure 1. The rooted branch was about 2.4 m. tall, and at the point of attachment to the bole was 4.4 cm. in diameter. The roots were traced for a horizontal distance of 1.8 m. but their ends were not reached. The parent tree was 15.2 cm. in diameter.

It is well known that layering frequently occurs in various gymnosperms, for example, *Tsuga*, *Picea*, *Abies*, *Chamaecyparis*, *Thuja*, *Juniperus*, and *Taxus*. The rare occurrence of the phenomenon in *Pinus* can scarcely be explained on the basis of unfavorable habitat conditions since pines commonly occur in association with other gymnosperms which do layer. It would seem that the intolerance of most pines to shade may be an important consideration in this con-

FIG. 1.—Basal branch of *Pinus strobus* which has developed roots and assumed an upright habit.

nection. Because of intolerance, the basal branches of most pines may die before sufficient organic debris has accumulated to cover them partially and thereby create conditions favorable for rooting.

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1. COOPER, WM. S., Reproduction by layering among conifers. BOT. GAZ. **52**: 369–379. 1911.
2. MAYR, HEINRICH, Waldbau auf naturgesetzlicher Grundlage. Berlin. 1925.

CURRENT LITERATURE

Hundert Jahre Zellforschung. By L. ASCHOFF, E. KÜSTER, and W. J. SCHMIDT. Protoplasma-Monographien, Band 17. Berlin: Gebrüder Borntraeger, 1938. Pp. x+285.

This volume includes three of the many discussions of the cell theory, its history and development, whose appearance has marked the close of a century since SCHLEIDEN and SCHWANN announced the universal significance of cellular structure. KÜSTER treats the development of the theory of the plant cell (62 pages); SCHMIDT discusses the development of the theory of the animal cell and its present position and significance (101 pages); and ASCHOFF deals with VIRCHOW's cellular pathology (99 pages).

The space available has allowed only a summary review of the results of innumerable workers. In consequence, each paper has become somewhat of an essay presenting the views of the writer rather than an adequate presentation of varied views upon controversial topics. This is particularly true in KÜSTER's short outline of the study of the plant cell. Both he and SCHMIDT have given individual credit to the earlier investigators, omitting almost entirely the citations of those of later years. ASCHOFF, dealing with a more restricted field, treats it somewhat more fully.

While the contributions of the three authors are distinct, there is evidence of collaboration. Little repetition appears, and there seems to be general agreement upon matters of opinion. Inevitably many of the judgments expressed will be unacceptable to some readers. An illustration of a ready source of controversy is the emphasis laid by KÜSTER upon the importance of SCHLEIDEN's contribution. Some other discussions that have appeared within the last two years have insisted more strongly upon the knowledge of plant cells available before the appearance of SCHLEIDEN's rather dogmatic little *Beiträge zur Phytogenesis*. SCHMIDT seems to agree with KÜSTER's views on this point, although he necessarily devotes more attention to SCHWANN's study of the animal cell.

One result of the brevity and comparative lack of "documentation" in the volume is that it is easily readable. It will be of use to the non-cytologist who may wish a hurried view of an extensive field. It will not serve as a work of reference for one who wishes to dig below the surface.—C. E. ALLEN.

Plant Physiology. By BERNARD S. MEYER and DONALD B. ANDERSON. New York: D. Van Nostrand Co. 1939. Pp. 696. $4.50.

During the last two years elementary textbooks of plant physiology have appeared so frequently that this branch of botany now seems well supplied with texts. There is always room, however, for a superior work, and this volume leaves little to be desired. It is well written, accurate, clear, and for the most

part logically arranged. The only feature of the organization that seems open to criticism occurs in the presentation of respiration, in which aerobic respiration, the last half of the process, is treated before anaerobic respiration, the first half. There is a common but fallacious notion that students are more familiar with aerobic than with anaerobic phases of respiration, and that the better known processes should be treated first—proceeding from the known to the unknown. As a matter of fact students are just as ignorant of aerobic processes as they are of anaerobic; and in presenting aerobic respiration first, one loses the continuity of the process. Moreover, all doubts of the continuous nature of anaerobic and aerobic processes have been swept away by recent investigations.

There are thirty-seven chapters, the earlier of which deal with such fundamentals as properties of solutions, interfacial phenomena, colloidal systems, properties of gels and sols, osmosis, imbibition, permeability, etc. Then the physiological processes are considered in logical order, followed by chapters that deal with the organism as a whole, growth, hormones, correlations, periodicity, and movements of adjustment.

There are 151 well-chosen text figures, and good bibliographies at the close of each chapter for supplementary reading. The work seems to present a satisfactory treatment of the subject as it is understood at the present time. Teachers and students should enjoy using a text that is so well balanced, so accurate and so well arranged.—C. A. SHULL.

Moss Flora of North America North of Mexico. Vol. I, Part 3. By A. J. GROUT. Newfane, Vermont: Published by the author, 1938. Pp. 137–192. Pls. 22. $2.50.

This part includes the families Encalyptaceae, Buxbaumiaceae, and Pottiaceae. (For reviews of previous parts see BOT. GAZ. **88**:111; 1929. **93**:110; 1932. **96**:578; 1935.) The first family is revised by SEVILLE FLOWERS, with a key to sterile specimens of *Encalypta* contributed by the editor. The genera *Pleurochaete* and *Tortella* (Pottiaceae) were contributed by INEZ M. HARING. WILLIAM C. STEERE furnished the revision for *Barbula* and *Didymodon*. All other genera have been revised by the editor. Especially noteworthy is the trend to synonymize species of genera commonly considered to be difficult taxonomically. In the revision of *Barbula* more than seventy species are reduced to eighteen, and one is described as new. Type specimens were studied in many instances. As in previously issued parts, the plates constitute nearly half the publication.—P. D. VOTH.

Anatomie der Vegetationsorgane der Pteridophyten. By Y. OGURA. Handbuch der Pflanzenanatomie. By K. LINSBAUER. II. Abt., Band VII, 2. Teil: Archegoniaten, B. Berlin: Gebrüder Borntraeger, 1938. Pp. viii+476. Illustrated. Rm. 60.

This volume is a summary of all published anatomical investigations on the vegetative organs of the Pteridophyta up to 1935. The general part is devoted

to the stem, leaf (including sporophyll and leaf apex), and root. All Pteridophyta are subdivided in the second part, where their anatomy is adequately discussed, into the Aphyllata (Psilophytean forms), the Microphyllata, and the Macrophyllata. Arrangement of smaller taxonomic groups within these subdivisions follows accepted modern interpretations. Although relatively few of the 382 figures are original, the illustrations are to the point and well chosen. More than 1200 literature citations are listed. There are indices for authors, for scientific names of plants, and for subjects. This book will serve as an excellent source for anatomical information.—P. D. VOTH.

Applied Silviculture in the United States. By R. H. WESTVELD. New York: John Wiley & Sons, 1939. Pp. vii+567. Figs. 102.

The entire United States is divided into eighteen regions characterized by their type of forest vegetation. Each is treated as a unit with reference to its physiographical and ecological features, past practices, and suggestions for future development, maintenance, and management. There are many good, pertinent illustrations and a fairly comprehensive bibliography at the close of each chapter.—E. J. KRAUS.

The Leguminous Plants of Wisconsin. The Taxonomy, Ecology, and Distribution of the Leguminosae Growing in the State Without Cultivation. By NORMAN C. FASSETT. Madison, Wisconsin: University of Wisconsin Press, 1939. Pp. xiii+157. Illustrated. $3.00.

Ninety-seven species and varieties belonging to twenty-seven genera of Leguminosae have been reported. Individuals of these species were studied in the field as well as in numerous herbaria, so that this attractive publication is the result of years of study. The chief artificial keys are based on vegetative characters; additional keys are based on flower, fruit, and seed differences. Every species and variety is discussed, and there is a critical chapter on the epidermal outgrowths of the plants. Distribution maps are included. Excellent photographs, some showing the vestiture of leaves and stems, increase the value of the book.—P. D. VOTH.

Keys to the Phyla of Organisms Including Keys to the Orders of the Plant Kingdom. By FRED A. BARKLEY. Missoula, Montana: Associated Student's Store, 1939. Pp. iv+39. 75¢.

These keys are designed to aid in placing unknown non-nucleated organisms, the Protista (Euthallophyta and Protozoa), and all remaining plants into the right orders; and the remaining animals into the respective phyla. An outline of the classification used forms a concise list of the main divisions of living and fossil organisms and indicates that the author is in agreement with many of the recent publications on classification. The glossary is sufficiently detailed to aid in the use of the keys. A selected list of references is appended.—P. D. VOTH.

Form- und Stoffwechsel der Golgi-Körper. By G. CHR. HIRSCH. Protoplasma-Monographien, Band 18. Berlin: Gebrüder Borntraeger, 1939. Pp. xi+394.

The extensive literature dealing with Golgi material in animal cells is reviewed and criticized in the light of the author's conception of its history, nature, and function. HIRSCH's conclusions are briefly as follows:

While it is always possible to distinguish mitochondria from Golgi bodies, there is nevertheless an intimate relation; either products of mitochondria are transformed directly into Golgi material or materials or enzymes produced by mitochondria function in the organization of Golgi material. The latter appears first as a "pre-substance," usually as structureless, irregular or rounded bodies which may be aggregated into a network. From the substance in this metabolically inactive state arise the active "Golgi systems," lying either isolated or in a localized region of the cell. The often-described appearance of a network at this stage is, according to HIRSCH, an artifact due to the adsorption of osmium tetroxide or silver nitrate on the surfaces of closely aggregated but distinct bodies. Each system becomes differentiated into an external portion still staining deeply with osmium or silver and an internal portion which is stained more lightly if at all. From the internal portion develops the "product," a secreted substance characteristic of the particular type of cell concerned. The "product" may be any one of a variety of substances, including albumins, slime, pigments, oils, fats, and hormones. Whether the Golgi systems involved in the formation of so diverse products are to be considered as belonging to a single category is left an open question. Also uncertain is the fate of the fragments of the external portion of a Golgi system when the "product" is freed. It is possible that these fragments may participate in a second sequence of pre-substance, system, product. At least it seems clear that most commonly the history of a single cell includes several such sequences.

The still very scattered discussions of supposed Golgi bodies in plant cells are cited rather uncritically. Thus GUILLIERMOND's identification of Golgi material with the plant vacuome, BOWEN's (tentative) identification with osmiophilic platelets, and WEIER's identification with plastids are quoted, with no suggestion that these three conceptions are contradictory and mutually exclusive. An interesting item is the author's recognition (p. 262) of *Polytrichum* as a fern. The usefulness of the book is enhanced by extensive and easily usable tabulations of observations and conclusions regarding Golgi bodies (real or supposed), and by a full bibliography which is arranged first by topics (chiefly according to organs or tissues concerned), and second alphabetically under authors' names.—C. E. ALLEN.